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14. ABSTRACT
We are using an innovative, quantitative assay for telomere DNA content (TC) developed and characterized by the PI, to test the hypothesis that TC predicts the likelihood of disease recurrence in women with ductal carcinoma in situ (DCIS). In Year One, we collaborated to determine whether TC measured in bulk DCIS tumor tissue is comparable to that measured in tumor epithelial cells purified by laser-capture microscopy. In 7/10 instances, TC in microdissected specimens was 72-112% of that in the undissected control. In Years Two and Three, we confirmed and extended these results in our own laboratory. TC in microdissected samples was compared to TC in unfractionated samples; in 10/10 instances, TC in the microdissected sample was 75-124% of that in the undissected (*i.e.* bulk) control.
These results confirm that it is not necessary to microdissect DCIS specimens prior to TC analysis. In Years One-Three, we measured TC in 75 normal breast, 126 DCIS and 657 breast tumor specimens. In Year Two, we used a Kaplan-Meier plot and log-rank test to show that low TC predicts a shorter survival interval. TC was not associated with ethnicity, menopausal status, or the expression of several other markers, including ER, PR, p53, Ki67, and Her2. In Years Three-Four, we demonstrated an association between TC, the extent of allelic imbalance and tumor stage. In Year Four, we obtained longer follow-up to confirm and extend these results. In summary, we have shown that (i) meaningful TC measurements can be obtained with bulk DCIS tissues, (ii) TC is associated with tumor stage and (iii) TC in DCIS is
associated with breast cancer-free survival.

15. SUBJECT TERMS
Ductal carcinoma in situ (DCIS), breast cancer, telomeres, prognosis, genomic instability

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Introduction

The widespread use of screening mammography has resulted in a ten fold increase in the incidence of ductal carcinoma in situ (DCIS) over the past twenty years, from 4800 cases in 1983 to more than 50,000 cases in the US in 2003 (1-3). This accounted for 18.3% of all newly diagnosed breast tumors, and 23% of newly diagnosed breast tumors in women 40-49 years of age in 2003 (2,3). However, the fraction of women with DCIS who eventually progress to invasive breast cancer is small (4-6). A Danish autopsy study found that 25% of women had *in situ* carcinomas, including DCIS, at their death, although the lifetime risk of developing breast cancer during the same period was only 1% (7). Similarly, only 32% of women whose DCIS was misdiagnosed as normal, and so did not receive treatment, went on to develop invasive carcinoma within 30 years of their biopsy (8,9). Less than 2% of women with DCIS die from breast cancer within ten years of diagnosis (10). Taken together, these results imply that up to two-thirds of women with DCIS would not progress to invasive cancer, even without treatment. Unfortunately, currently available prognostic markers are unable to discriminate between DCIS that will and will not progress; therefore, many women receive aggressive treatments that may be unnecessary. Currently, 97.5% of women with DCIS in 1999 had some type of surgery, of which 28% had radical mastectomies (11). In the March, 2004 issue of the Journal of the National Cancer Institute, Baxter et al (11) wrote: "*The potential for preventing invasive breast cancer is important, yet the risk for over treatment is a clinically significant concern*". In an accompanying editorial, Dr. Morrow comments on barriers to developing meaningful therapeutic guidelines (12). She writes:

"*The first of these is our inability to identify which DCIS lesions will progress to invasive carcinoma, and in what time interval. Conventional prognostic factors, such as patient age and tumor grade, subtype, and size, provide information on the time course of local recurrence and the magnitude of risk reduction achieved with radiotherapy, but these factors do not identify those women who will have a disease recurrence with potentially life-threatening invasive cancer. Efforts to identify a molecular signature for DCIS lesions that will recur as invasive carcinoma are of enormous interest…*"

Using an innovative, quantitative assay for telomere DNA content (TC) developed and characterized by the PI (13-17), we have recently shown that TC in tumor tissue is associated with cancer-free survival in women with breast cancer (18). The purpose of this investigation is to determine whether TC can be used similarly to predict the likelihood of disease progression in women with DCIS.

Body

Tasks: The agreed upon tasks to be completed during the first year of the IDEA Award were:

Aim One: We will compare TC measured in bulk DCIS tumor tissue to TC measured in tumor epithelial cells that have been stripped of stromal cells and connective tissue by laser-capture microscopy.

Task 1	Months 1-6	Obtain 30 random archival specimens of DCIS.
Task 2	Months 2-12	Extract DNA from bulk DCIS samples and measure telomere DNA content (TC). Divide study group into thirds, based on TC.
Task 3	Months 4-12	Use laser capture microscopy to purify tumor epithelial cells from stromal and cells and connective tissues in the 10 samples comprising the middle third of the study group.
Task 4	Months 6-12	Extract DNA from purified epithelial cells and measure TC. Compare TC in bulk DCIS tissue to purified tumor epithelial cells.

Aim Two: The data from aim one will be used to guide the study design for the second aim, in which we will perform a retrospective study of the association between TC and time to disease recurrence in women with DCIS.

Task 5	Months 1-6	Design search parameters for NMTR database and identify 120 members of study group.
Task 6	Months 1-6	Establish data base of patient records
Task 7	Months 3-24	Obtain tissue blocks and cut new sections.
Task 8	Months 12-30	Extract DNA from 120 bulk specimens of DCIS or enriched epithelial cells, depending on the outcome of Aim One, and measure TC.
Task 9	Months 12-30	Compare TC to disease recurrence status
Task 10	Months 30-36	Draft and submit manuscripts

Progress Relative to Tasks:

Tasks 1 and 2 have been completed. Retrospective DCIS cases (N=27) were obtained from the Surgical Pathology Department at the University of New Mexico Hospital (UNMH). All cases were diagnosed in 2004 and 2005 and the presence of DCIS was confirmed by Dr. Nancy Joste, Director of Anatomic and Cyto-pathology. Four 25 μm sections of the paraffin-embedded, formalin-fixed (FFPE) tissue were obtained and DNA was isolated using standard techniques. TC was analyzed for all the samples and the TC distribution of the samples is plotted in Figure 1. TC in these samples ranged from 58-218% of the placental DNA control with a mean TC value of 145%. The middle 10 cases, contained within the dotted lines (TC range: 129-155% of placental DNA control), were used in subsequent analysis.

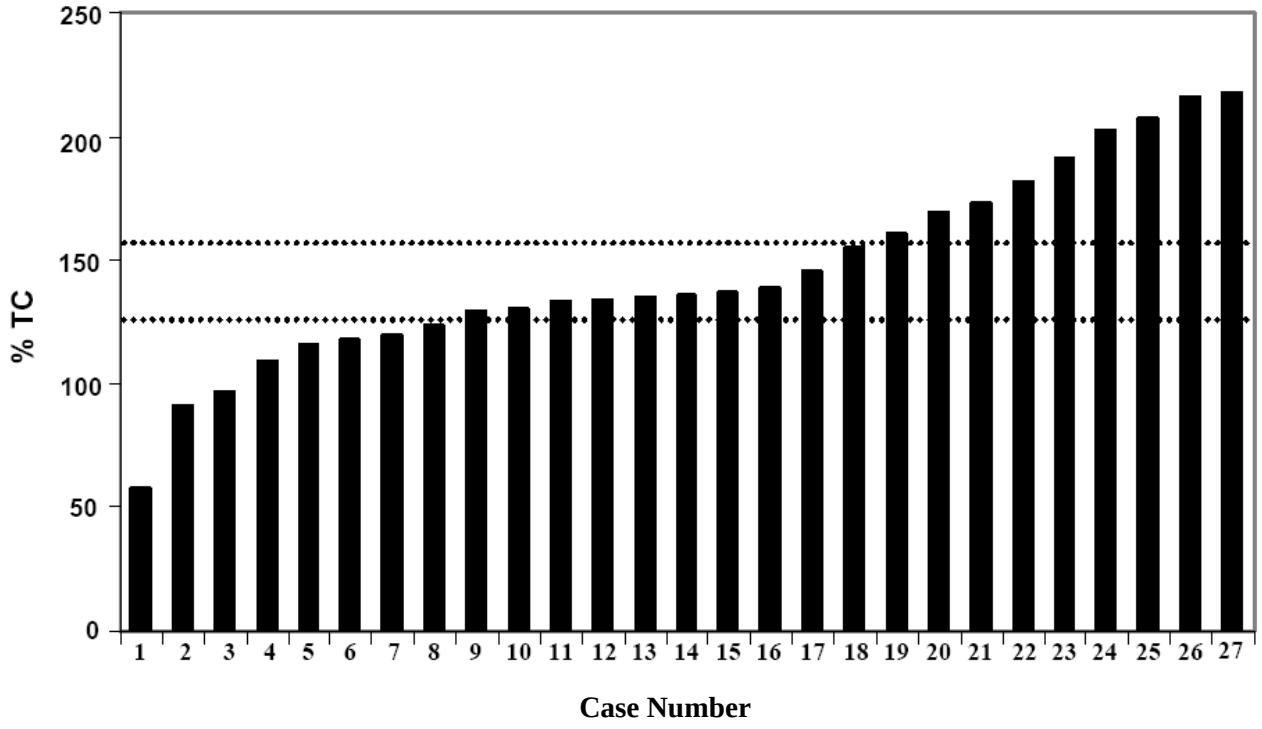

Figure 1. Distribution of Telomere DNA Contents (TC) in 27 Specimens of Bulk DCIS tissues. *Each DCIS specimen is shown on the x-axis. TC in each specimen is shown on the y-axis and is expressed as a ratio of TC in a placental DNA control, which is defined as 100%. The two dotted lines represent the upper (155%) and lower (129%) limits for the middle 10 cases used in subsequent analyses.*

Tasks 3 and 4 are complete. In Year One, to minimize possible delays resulting from tissue acquisition, we collaborated with Dr. Colleen Fordyce to measure TC in 10 pairs of bulk DCIS tissue obtained from her laboratory. In 7/10 instances, TC in the microdissected specimens was 72-112% of

that in the undissected control. The difference in TC in bulk and microdisected tissue was relatively constant (median 85%), implying that it would not be necessary to microdissect or otherwise fractionate DCIS specimens prior to TC analysis.

In Year Two, we obtained and characterized DCIS tissues that allowed us to confirm these results in our own laboratory. The 10 cases of DCIS from Figure 1 that comprise the middle tertile of TC values (Range: 129-155% of the placental DNA control) were further analyzed. Ten consecutive 10 μm sections were obtained for these samples with the odd numbered sections fixed to slides and the even numbered sections pooled together for bulk TC analysis. The samples for the bulk TC were again analyzed and compared to the original TC values. TC in the initial bulk analysis and the subsequent bulk analysis were not statistically different (p=0.762) and, in fact, yielded nearly identical results (median difference 2%). In Year Three, the odd numbered sections fixed to glass slides were microdissected to isolate only cells with DCIS and TC was determined. TC in the microdissected DCIS tissue samples was compared to TC in the paired bulk analysis (Figure 2). There was virtually no difference in TC in bulk and microdisected (*i.e.* bulk) tissue (median difference 5%, range -25% to +24%). These findings, in conjunction with the findings in Year One, demonstrate it is not necessary to microdissect DCIS tissue prior to TC analysis.

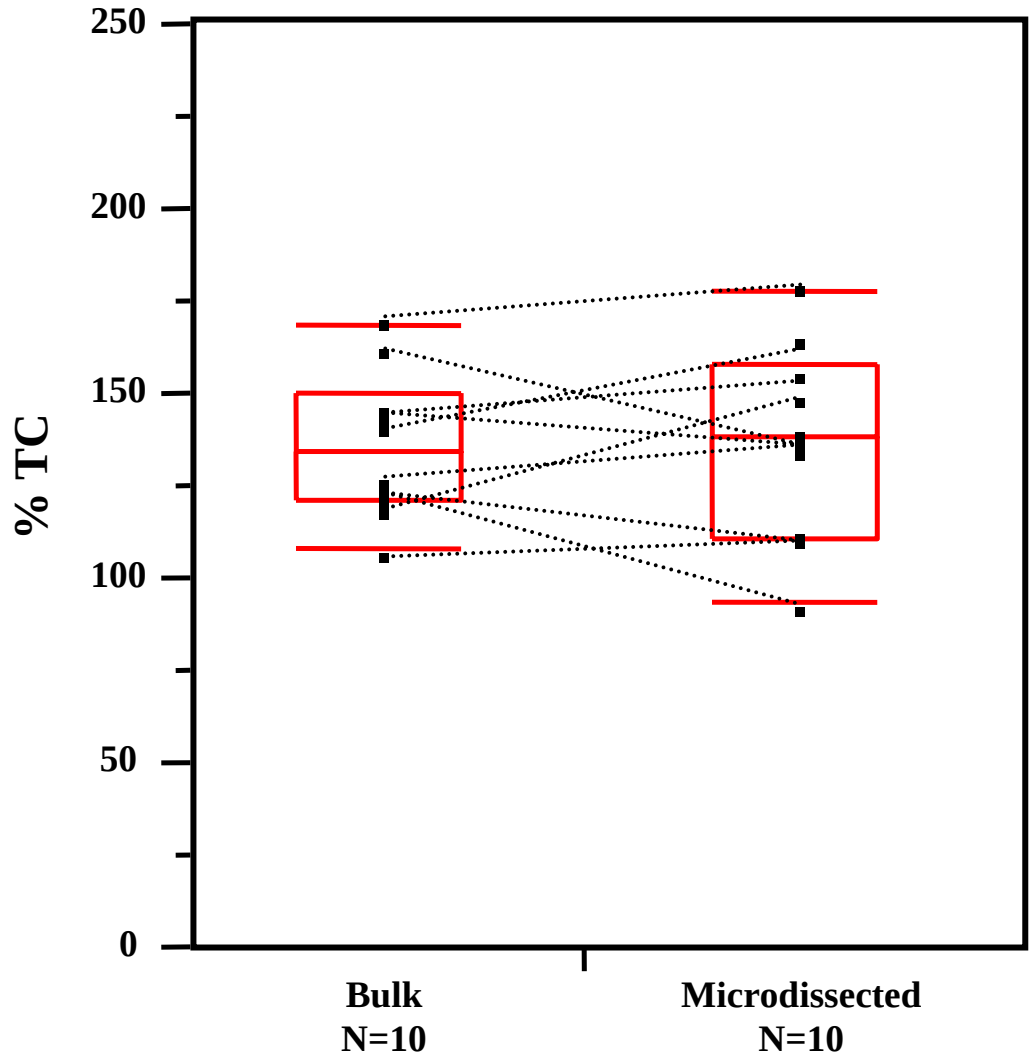

Figure 2. Distributions of Telomere DNA Contents (TC) in Ten Pairs of Microdissected and Bulk DCIS Tissues. *TC in each specimen is shown on the y-axis and is expressed as a ratio of TC in a placental DNA control, which is defined as 100%. The line across the middle of each box shows the group median and the quartiles (25th and 75th percentiles) as its ends. The 10th and 90th quantiles are*

shown as lines above and below the box. TC values of the individual matched samples are connected by dotted lines. TC was measured as in Figure 1.

Tasks 5 and 6 have been completed.

Tasks 7 and 8 have been completed. In Years One, Two and Three, DNA was purified and TC measured from 126 specimens of DCIS. For comparison, we also measured TC in 75 specimens of normal breast tissue derived from reduction mammoplasty, and 657 specimens of breast cancer tissues TNM stages I-III (Figure 3). Non-parametric Rank Sums (Kruskal-Wallis) test demonstrates a statistically significant difference between the mean TC in DCIS tissue and all TNM stages (I-III) of breast cancer tissues ($p<0.0001$ for each).

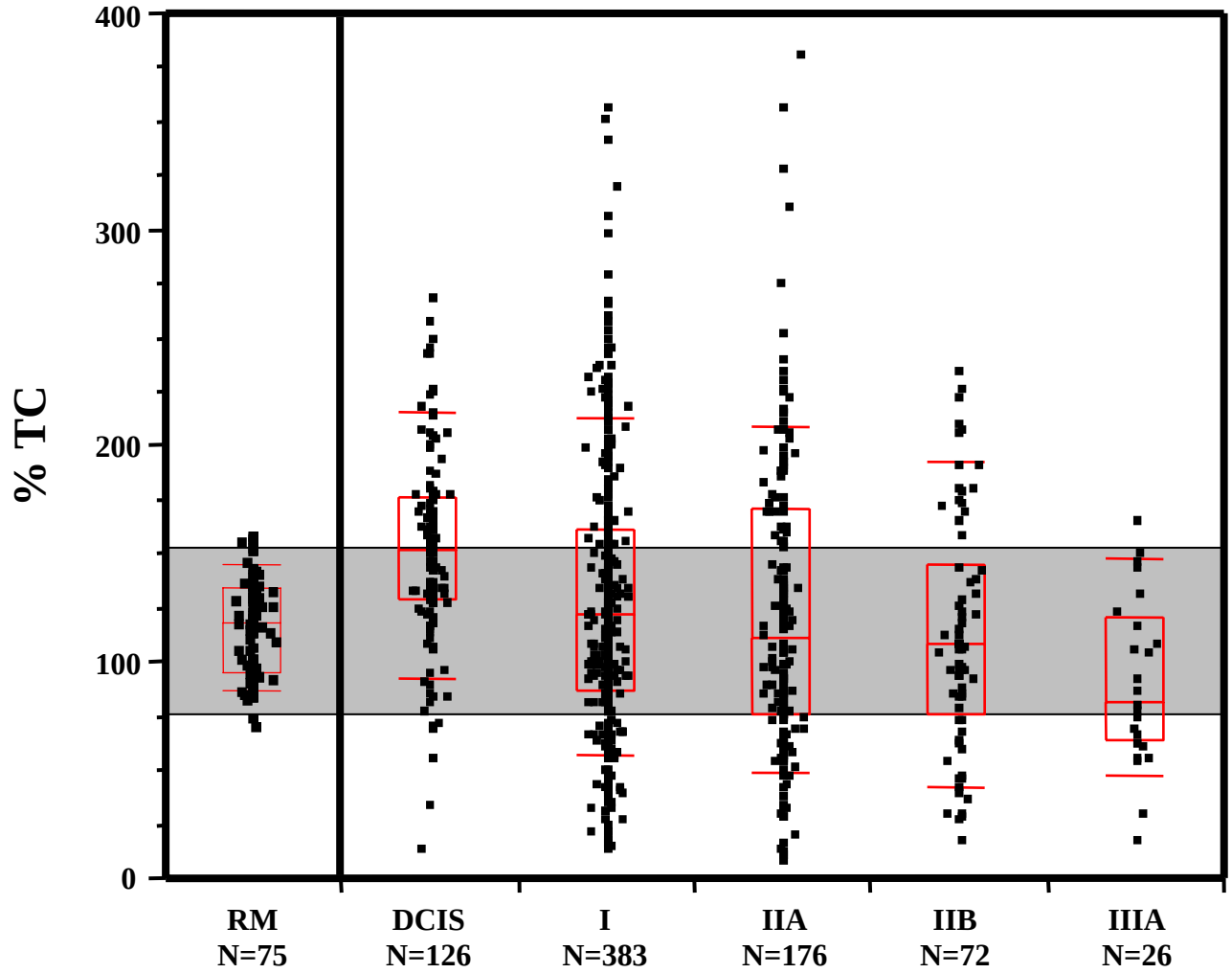

Figure 3. Distributions of Telomere DNA Contents (TC) in Normal Breast Tissues, DCIS, and Invasive Breast Tumors. *TC in each specimen is shown on the y-axis and is expressed as a ratio of TC in a placental DNA control, which is defined as 100%. The number of specimens in each tissue set (N) is indicated. The line across the middle of each box shows the group median and the quartiles (25th and 75th percentiles) as its ends. The 10th and 90th quantiles are shown as lines above and below the box. The gray shaded area indicates 95% of TC measurement for all normal specimens (75-154% of standard control DNA).*

Task 9 has been completed. A large fraction of the samples shown in Figure 3 (N=530) was collected as a part of the prospective, population-based Health, Eating, Activity and Lifestyle (HEAL) Study. The HEAL multi-center study was designed to evaluate the association between body composition, hormones, diet, physical activity, and prognosis over time for non-Hispanic white, Hispanic, and African-American women ascertained through the Surveillance, Epidemiology, and End Results (SEER) registries. In Years Two and Three, we completed TC analysis and retrieved coded patient data, stripped of all personal identifiers, as approved by the University of New Mexico Human Research Review Committee. The cohort was initially divided into sixths, the survival interval for each group was calculated, and the results were evaluated for statistical significance by log-rank analysis. Groups with statistically indistinguishable survival intervals were combined and the process was repeated until only groups with significantly different survival intervals remained. Using this process, the cohort was stratified into two TC groups: low TC was defined as ≤ 200% in the placental DNA control, and high TC was defined as > 200% of TC in the placental DNA control, respectively.

We next extracted the data for the subset of the HEAL cohort with DCIS (N=97). The mean age and follow-up of cohort members were 57.1 (Range: 36-89; SD: 11.7) and 6.9 (Range: 2.8-9.0; SD: 1.0) years, respectively. At the time of analysis, 96% of the cohort members were alive. Additionally, 89% of the cohort members were free of disease, either at time of analysis or at time of their non-breast cancer related death. We evaluated the prognostic value of the low (N=80) and high (N=17) TC groups in predicting breast cancer-related, adverse event-free survival interval. An adverse event was defined as death due to breast cancer, breast cancer recurrence or development of a new primary breast tumor. Eleven breast cancer-related adverse events had occurred by the time of the analysis. A Kaplan-Meier plot and log-rank test (Figure 4) demonstrated a trend, although not statistically significant, showing low TC predicts a shorter survival interval (p=0.111). All of the 11 cases with a documented adverse event were in the low TC group. TC was not associated with ethnicity, menopausal status, or the expression of several other markers, including estrogen receptor, progesterone receptor, p53, Ki67, and Her2.

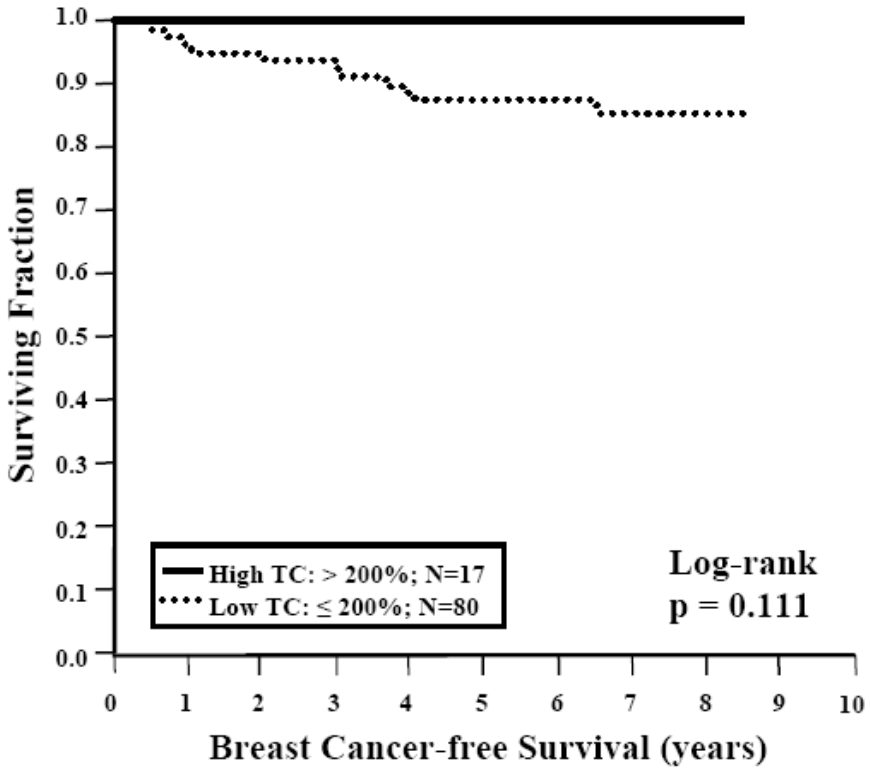

Figure 4. Breast Cancer-free Survival Interval by Telomere DNA Content in 97 DCIS cases. *The set of DCIS cases was divided into two groups based on the low (N=80) and high (N=17) TC cutoff (200%*

of placental DNA control). Breast cancer-free survival interval in years, is shown on the x-axis. The surviving fraction is shown on the y-axis. Subjects were censored at the time lost to follow-up. The log-rank test was used to test the significance (p) of the differences in the group's survival intervals. N represents the number of subjects in each group.

At the conclusion of Year Four, The New Mexico Tumor Registry provided updated follow up data on subjects in the HEAL study. This new data, which for some patients provides up to 10 years of follow up data, has been added to our database and the relationship between TC and breast cancer-free survival in women with DCIS is being reanalyzed.

In Years Three and Four, we evaluated the link between telomere dysfunction and the generation of allelic imbalance (AI) in the progression of breast cancer. We assessed alterations in TC and the extent of AI in a continuum of breast tissues ranging from histologically normal tissue derived from reduction mammoplasty, to ADH, DCIS and invasive carcinomas ranging from Stage I-IIIA. The extent of AI was determined using a straight-forward, economical, and high-throughput method recently developed by our laboratory. This method evaluates AI in a panel of 16 randomly selected microsatellite markers (*i.e.* markers with no known relationship to breast cancer) thereby preventing measurement bias by selection of genes whose products are involved in tumorigenesis. As shown in Figure 5, we demonstrate that changes in AI, which exceed values typically observed in normal tissues, increases along the continuum of breast disease; however, it plateaus in DCIS without further increase in the invasive carcinomas. These results were replicated in two independent sets of breast tumor tissues, demonstrating that DCIS lesions have a similar extent of genomic instability as invasive carcinomas. These data are consistent with (i) the proposed relationship between dysfunctional telomeres and genomic instability, (ii) the notion that invasive carcinomas evolve from or in parallel with DCIS, and (iii) the resultant hypothesis that tumors, including DCIS, with the shortest telomeres have the most unstable genomes and, consequently, the greatest probability of containing cells with the phenotypes associated with disease recurrence. In summary the data support the idea that TC is a suitable prognostic marker for invasive carcinomas, and most importantly, for *in situ* carcinomas.

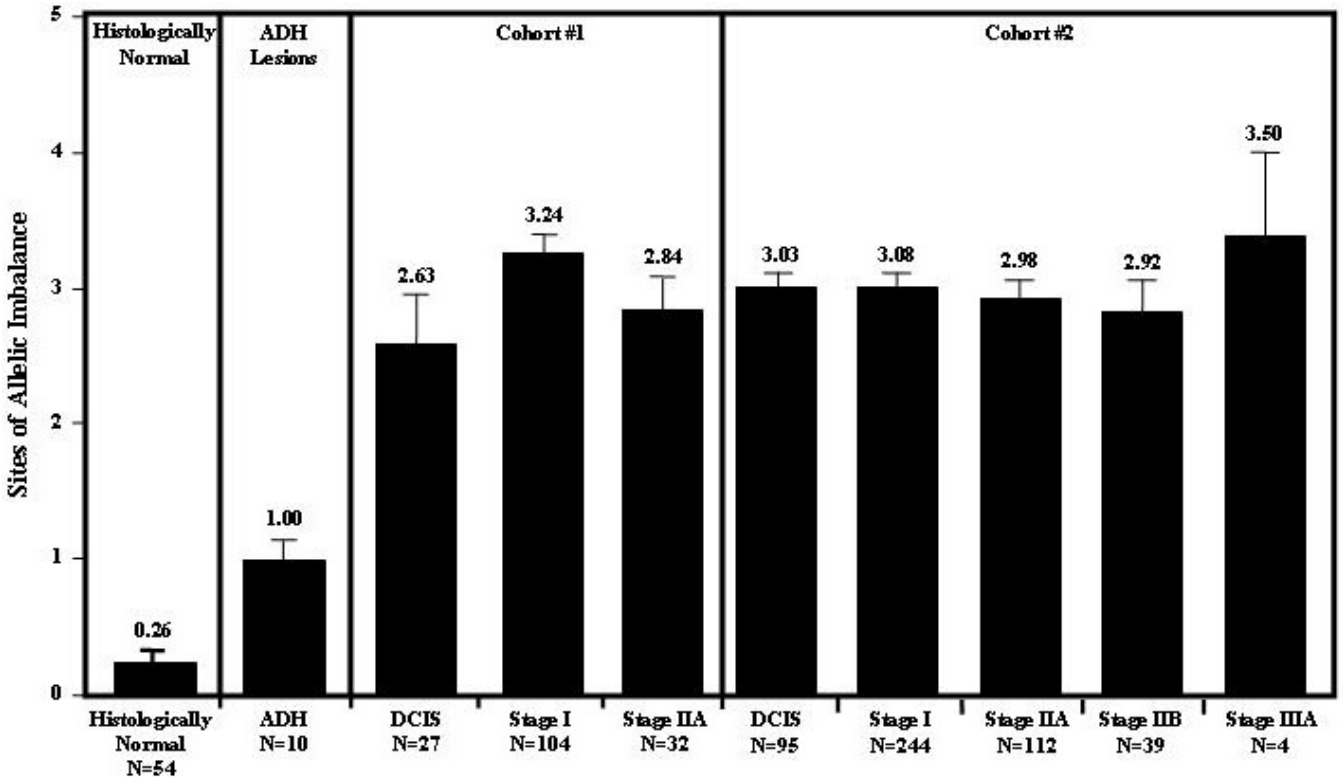

Figure 5. Extent of allelic imbalance (AI) in histologically normal breast tissues derived from reduction mammoplasties, ADH lesions, and two independent cohorts of DCIS lesions and invasive breast carcinomas (Stage I-III). *The numbers of tissues analyzed are indicated (N). The bars indicate the mean number of unbalanced loci (shown for each group) +/- standard errors. Abbreviations: ADH: atypical ductal hyperplasia; DCIS: ductal carcinoma in situ.*

Task 10 has been completed. Three manuscripts resulting from this project have been published in peer-reviewed journals (18-20, appendix).

KEY RESEARCH ACCOMPLISHMENTS FOR YEAR FOUR

- Additional tissues were procured.
- Additional clinical and patient follow data was obtained for 97 members of HEAL Cohort.
- Breast cancer-free survival data in women with DCIS was analyzed as a function of telomere DNA content.
- The extent of allelic imbalance was assessed in normal, ADH, DCIS and invasive breast specimens to confirm the relationship between telomere dysfunction and genomic instability.

REPORTABLE OUTCOMES FOR YEAR FOUR

- DNA banks from 75 specimens of normal breast tissue, 126 specimens of DCIS and 657 specimens of breast tumor tissues have been produced.
- A corresponding database has been produced that contains anonymous patient histories, including age at diagnosis, ethnicity, treatments, tumor stage, estrogen and progesterone receptor status, tumor size, length of disease-free survival or date and cause of death and diagnosis; telomere content and allelic imbalance.
- Data from this investigation is included in two peer-reviewed publications (appendix) and five meeting abstracts.

CONCLUSIONS

Prior studies show that telomere DNA content (TC) is a novel and independent prognostic marker in breast tumors. The data obtained are consistent with the conclusions that: (i) meaningful TC measurements can be obtained with bulk DCIS tissues, (ii) TC is associated with tumor stage, (iii) invasive carcinomas evolve from or in parallel with DCIS and (iv) TC in DCIS is associated with breast cancer-free survival.

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Meeting Abstracts:

C.M. Heaphy, C.A. Fordyce, M. Bisoffi, J.L. Wyaco, N.E. Joste, A. Mangalik, K. Baumgartner, R. Baumgartner, W.C. Hunt and J.K. Griffith (2006) *Telomere content correlates with stage and prognosis in invasive breast cancer.* **1st Biennial National IDeA Symposium of Biomedical Research Excellence (NISBRE). Washington, D.C.**

C.M. Heaphy, K.B. Baumgartner, M. Bisoffi, R.N. Baumgartner and J.K. Griffith (2007) Telomere DNA content predicts breast cancer-free survival interval. **National Graduate Student Research Festival, National Institutes of Health. Bethesda, MD.**

C.M. Heaphy, K.B. Baumgartner, M. Bisoffi, R.N. Baumgartner and J.K. Griffith (2007) Telomere DNA content predicts breast cancer-free survival interval. **AACR Special Conference: Advances in Breast Cancer Research. San Diego, CA.**

C.M. Heaphy, K.B. Baumgartner, M. Bisoffi, R.N. Baumgartner and J.K. Griffith (2008) Telomere DNA Content Predicts Overall and Breast Cancer-free Survival Intervals. **Next Generation Sequencing Symposium, Santa Fe, NM.**

M. Bisoffi, C.M. Heaphy, K.B. Baumgartner, R. N. Baumgartner, and J. Griffith (2008) Telomere DNA Content Predicts Overall and Breast Cancer-free Survival Intervals. **2008 DOD Era of Hope Symposium, Baltimore, MD.**

M. Bisoffi, C.M. Heaphy, N.E. Joste, K.B. Baumgartner, R. N. Baumgartner, and J. Griffith (2008) Assessing the Extent of Allelic Imbalance in Human Breast Tissue Using a Multiplex PCR System. **2008 DOD Era of Hope Symposium, Baltimore, MD**.

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Preclinical study

Telomere content correlates with stage and prognosis in breast cancer

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Key words: breast cancer, genomic instability, metastasis, prognosis, telomere, TNM staging

Summary

Purpose. To evaluate the hypothesis that telomere DNA content (TC) in breast tumor tissue correlates with TNM staging and prognosis.

Experimental design. Slot blot assay was used to quantitate TC in 70 disease-free normal tissues from multiple organ sites, and two independent sets of breast tumors containing a total of 140 samples. Non-parametric Rank–Sums tests, logistic regression and Cox proportional hazards models were used to evaluate the relationships between TC and tumor size, nodal involvement, TNM stage, 5-year survival and disease-free interval.

Results. TC in 95% of normal tissues was 75–143% of that in the placental DNA standard, whereas only 50% of tumors had TC values in this range. TC was associated with tumor size ($p = 0.02$), nodal involvement ($p < 0.0001$), TNM stage ($p = 0.004$), 5-year overall survival ($p = 0.0001$) and 5-year disease-free survival ($p = 0.0004$). A multivariable Cox model was developed using age at diagnosis, TNM stage and TC as independent predictors of breast cancer-free survival. Relative to the high TC group (>123% of standard), low TC (<101% of standard) conferred an adjusted relative hazard of 4.43 (95% CI 1.4–13.6, $p = 0.009$). Receiver operating characteristic curves using thresholds defined by the TC distribution in normal tissues predicted 5-year breast cancer-free survival with 50% sensitivity and 95% specificity, and predicted death due to breast cancer with 75% sensitivity and 70% specificity.

Conclusions. TC in breast cancer tissue is an independent predictor of clinical outcome and survival interval, and may discriminate by stage.

Introduction

It is estimated that in the US in 2005 more than 200,000 women were diagnosed with breast cancer, and approximately 40,000 women died from this disease. Micrometastasis (metastatic cells that have escaped the primary tumor, but are currently undetectable) are a primary cause of breast cancer recurrence and mortality. Although TNM (Tumor size-Nodal involvement-Metastasis) is among the most informative of current prognostic markers for breast cancer [1–2], it often fails to discriminate between women who will have favorable and poor outcomes [1–5]. Thus, it is important to develop new markers that accurately predict the likelihood of breast cancer recurrence at the time of diagnosis.

Nearly a century ago, Boveri proposed that cancer resulted from altered genetic material. It is now widely accepted that genomic instability – the amplification, loss or structural rearrangement of a critical gene or genes – occurs in virtually all cancers [6]**.** The phenotype of a tumor is a reflection of its gene expression. Therefore, mechanisms that generate genomic instability, and thereby alter gene expression, play direct roles in tumor progression, including the development of aggressive tumor phenotypes like micrometastasis. Telomere dysfunction is one mechanism of generating genomic instability [7–9]. Telomeres are nucleoprotein complexes that protect the ends of eukaryotic chromosomes from degradation and recombination [10–12]. Due to incomplete replication, telomeres are shortened during each round of cellular replication [13]. Telomere shortening

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may also be a consequence of double-strand DNA breaks, or changes in either the expression or function of any of the numerous proteins required for telomere maintenance [14–16]. Critically shortened, dysfunctional telomeres are prone to chromosome fusion and breakage [17], and in normal somatic cells lead to p53-dependent senescence and apoptosis [18]. However, these mechanisms are inactivated in cancer cells, for example, through p53 and Rb mutations. The direct relationship between dysfunctional telomeres, genomic instability and altered gene expression implies that tumors with the shortest telomeres have the most unstable genomes and, consequently the greatest probability of aberrant gene expression. Likewise, tumors with the longest telomeres would be expected to have fewer genomic alterations, and therefore, lower probability of containing cells with the phenotypes associated with disease recurrence. Accordingly, we and others have postulated that the mean telomeric DNA length in a tumor may provide a surrogate for phenotypic variability and therefore have prognostic potential in tumors [19–21].

There have been several investigations of the relationship between telomere length, or its proxies, and outcome in cancer. The most well characterized of these are in hematological cancers where it has been shown that telomere loss is associated with decreased survival in multiple types of leukemia and myeloma [22–24]. However, there have been few investigations of the prognostic potential of telomere length in solid tumors, which account for the majority of cancer incidence. Primarily, this is because the limited quantity and poor quality of DNA that is typically recovered from archival tissues precludes the use of Southern blotting techniques for the determination of telomere length.

To circumvent these problems, we previously described an alternative approach for measuring telomere length in genomic DNA obtained from fresh, frozen and, most importantly, paraffin-embedded tissues up to 20 years old [25,26]. The content of telomere DNA sequences (TC) in a DNA sample is titrated by hybridization with a telomere specific probe, and then normalized to the quantity of total genomic DNA in the same sample, thus controlling for the differences in DNA ploidy that are frequent in solid tumors. Our previous studies have shown that TC measured by this method is directly proportional to mean telomere length determined by Southern blotting [25]. Thus, TC is a proxy for telomere length and not affected by TTAGGG sequences outside the telomere. However, in contrast to Southern blotting, the TC assay can be performed with as little as 5 ng of genomic DNA and fragmented DNA less than 1 KB in length [25,26]. Therefore, the TC assay is particularly well-suited for analysis of retrospective studies of archival specimens from subjects with known outcomes.

Using this method, we previously demonstrated that reduced TC is associated with metastasis to lymph nodes in breast cancer [19]. More recently, we reported that TC was an independent predictor of time to prostate cancer recurrence (RH = 5.02) [20]. Short telomeres have also been associated with poor outcomes in neuroblastomas [27] while very long telomeres are a positive prognostic indicator in glioblastoma multiforme [28]. Collectively, these data imply that the extent of telomere loss or gain in tumors may have wide potential as a prognostic marker. However, this conclusion must be considered provisional, as prior studies often were based on small numbers of samples, highly selected patient populations and limited follow-up data using multiple clinical endpoints. In addition, the criteria for defining ''long'' or ''short'' telomeres are usually relative, and the relationships between telomere lengths in tumors and true disease-free tissue are often undefined.

In the current investigation, we have used the TC assay to define a normal range of telomere DNA content in breast and other tissues from multiple sites in healthy donors, compared this range to the distribution of TC measured in breast tumor tissues, and evaluated the relationships in breast tumor tissues between TC and TNM stage (and its individual components), 5-year breast cancer survival, and breast cancer-free survival interval following surgical excision of breast carcinoma.

Materials and methods

Tissue samples

Four independent sets of human breast tissues were used in this study. The first set (1982–1993) was comprised of 77 archival frozen and paraffin-embedded breast tumor tissues from women with either invasive ductal or lobular carcinomas who had radical mastectomies ($N = 63$), breast sparing surgery ($N = 11$) or unspecified surgeries ($N = 3$) between 1982 and 1993. The second set (1996–1999) was comprised of 63 archival paraffin-embedded breast tissues from a randomly selected subset of women participating in the population-based Health, Eating, Activity and Lifestyle (HEAL) Study [29]. These women were diagnosed with ductal carcinoma *in situ* (DCIS), invasive ductal carcinomas or invasive lobular carcinomas, and had radical mastectomies ($N = 11$) or breast sparing surgery ($N = 52$) between 1996 and 1999. Clinical data on breast tumors (Tables 1, 2) were ascertained by the New Mexico Tumor Registry (NMTR), a member of the Surveillance, Epidemiology, and End Results (SEER) Program of the National Cancer Institute. TNM stage was assigned using the 2002 revised criteria [30]. This study was approved by the University of New Mexico (UNM) Human Research Review Committee.

The third set was obtained from the National Cancer Institute Cooperative Human Tissue Network (Nashville, TN) and contained disease-free breast tissue from women who had reduction mammoplasty (RM). The fourth set included matched tumor and histologically normal breast (HNB) tissues collected at sites 5 cm from

Table 1. Characteristics of tumor tissues

Set[a]	*N*	Size (mm)					Node involvement			TNM stage						Number of deaths at 5-years of follow up	
		Median	Mean	Range	Q1	Q3	Yes (%)	No (%)	NA (%)	0 (%)	I (%)	IIA (%)	IIB (%)	IIIA (%)	IIIB (%)	IV (%)	NA (%)	Total (%)	Breast cancer related (%)[b]
1982–1993	77	30	34	8–80	20	49	51 (66)	25 (33)	1 (1)	0 (0)	11 (14)	20 (26)	25 (32)	16 (21)	1 (1)	2 (3)	2 (3)	27 (35)	17 (22)
1996–1999	63	14	16	0–65	7	20	15 (24)	41 (65)	7 (11)	11 (17)	32 (51)	12 (19)	6 (9)	1 (2)	0 (0)	0 (0)	1 (2)	9 (14)	0 (0)
Combined	140	20	26	0–80	12	34	66 (47)	66 (47)	8 (6)	11 (8)	43 (31)	32 (23)	31 (22)	17 (12)	1 (1)	2 (1)	3 (2)	36 (26)	17 (12)

Abbreviations: *N*, number of specimens; Q1, Q3, first and third quartile (the difference between Q1 and Q3 is the inter-quartile range, or IQR); NA, not available.

[a]Tissue sets are described in the Materials and methods section.

[b]Of the 10 subjects who died from causes other than breast cancer, six died from other cancers, and one each from dementia, hypertension, pulmonary embolism, and unknown causes.

the visible tumor margins from women receiving full mastectomies at UNM Hospital in 2003 and 2004. To determine the extent to which TC differed as a function of age, tissue of origin and disease-status, buccal cells (BUC) were obtained from healthy male and female college student volunteers and peripheral blood lymphocytes (PBL) were obtained from women previously diagnosed with breast cancer.

Histological review

Paraffin-embedded and frozen tissue sections were stained with hematoxylin and eosin and were examined microscopically. Tumor tissues typically contained from 75–100% tumor cells.

Determination of telomere DNA content (TC)

DNA was extracted from slides cut from frozen or paraffin-embedded tissue, and TC was measured as described [20,26]. Briefly, DNA was isolated from frozen or paraffin-embedded tissues, and blood samples using Qiagen DNeasy Tissue kits (Qiagen, Valencia, CA) and the manufacturer's protocols. DNA was denatured at 56 °C in 0.05 M NaOH/1.5 M NaCl, neutralized in 0.5 M Tris/1.5 M NaCl, and applied and UV cross-linked to Tropilon-Plus blotting membranes (Applied Biosystems, Foster City, CA). A telomere-specific oligonucleotide, end-labeled with fluorescein, (5′-TTAG GG-3′)$_4$-FAM, (IDT, Coralville, IA) was hybridized to the genomic DNA, and the membranes were washed to remove non-hybridizing oligonucleotides. Hybridized oligonucleotides were detected by using an alkaline phosphatase-conjugated anti-fluorescein antibody that produces light when incubated with the CDP-Star substrate (Applied Biosystems, Foster City, CA). Blots were exposed to Hyperfilm for 2–10 min (Amersham Pharmacia Biotech, Buckinghamshire, UK) and digitized by scanning. The intensity of the telomere hybridization signal was measured from the digitized images using Nucleotech Gel Expert Software 4.0 (Nucleotech, San Mateo, CA). TC is expressed as a percentage of the average chemiluminescent signal from three replicate determinations of each tumor DNA relative to the average chemiluminescent signal in the same amount (typically 20 ng) of a reference DNA standard (placental DNA). DNA purified from HeLa cells, which have approximately 30% of the TC in placental DNA, and samples prepared without DNA served as positive and negative controls, respectively.

Statistical methods

We compared the distribution of TC for normal and tumor specimens and, within tumor specimens, by tumor size, nodal involvement, and TNM stage using schematic plots and the non-parametric Rank–Sums (Kruskal–Wallis) test. Logistic regression was used to model the fraction of tumors <2 cm in size, node

Table 2. Ages at tissue collection and telomere DNA contents in normal and tumor tissues

Set[a]	*N*	Age at tissue collection					Telomere DNA content (% placental DNA control)				
		Median	Mean	Range	Q1	Q3	Median	Mean	Range	Q1	Q3
Normal											
RM	20	30	29	15–48	21	36	126	127	114–158	120	132
HNB	12	53	49	26–61	39	59	101	101	70–128	79	124
PBL	12	NA	NA	NA	NA	NA	87	91	71–117	78	106
Buccal	26	NA	NA	NA	NA	NA	110	114	89–148	100	126
Combined	70	36	36	15–61	25	51	116	112	70–158	98	126
Tumor											
HNB Matched	12	53	49	26–61	39	59	57	59	24–108	42	69
1982–1993	77	48	52	31–88	42	60	108	109	36–247	77	126
1996–1999	63	56	59	32–85	48	72	136	148	31–359	98	177
Combined	152	53	55	26–88	45	65	110	121	24–359	76	146

Additional details are found in the text and the legend to Figure 1. Abbreviations: N: Number of specimens, Q1, Q3: first and third quartile (The difference between Q1 and Q3 is the interquartile range, or IQR). NA: Not available.
[a]Tissue sets are described in the Materials and methods section.

negative status, and at each TNM stage as a function of TC. The results of the logistic regression models are shown as plots of predicted values against TC. We investigated the association between survival and TC using Kaplan–Meier survival plots for three categories of TC, which were based on tertiles of the TC distribution in normal specimens. Death from any cause and death due to breast cancer were evaluated separately in the survival analyses. Cox proportional hazards models were used to control for the confounding effects of TNM stage and age. SAS version 9.1 and JMP (SAS Institute) were used for all analyses. *P*-values < 0.05 were considered to be significant.

Results

Telomere contents in normal tissues

Telomere content can be affected by several inherent properties, such as patients' ages and health status, and the organ sites from which the tissue specimens were collected. To evaluate the potential variability in TC arising from inherent properties of tissues, TC was measured in a diverse sampling of 70 specimens of normal tissue from multiple organ sites (Figure 1). Specimens included breast tissue obtained by reduction mammoplasty (RM); histologically normal breast tissues excised from sites 5 cm from the breast tumor margins (HNB), buccal cells from healthy, young men and women (BUC) and PBL from women with a prior diagnosis of breast cancer (PBL). As summarized in Table 2, median TC in HNB and PBL sets (101 and 87%, respectively) were approximately 30% lower than median TC in the RM and buccal specimens (126 and 110%, respectively). Similarly, the median ages for the donors of the HNB set (53 years) was almost twice the median ages of the donors of the RM samples (30 years). Although the ages of the volunteers contributing the BUC and PBL samples were not collected, the BUC samples were obtained from college students in their early 20s, while the PBL samples were obtained from a subset of a larger study group with a median age of 58 years. Thus, the results are consistent with the accepted view that telomere length in humans decreases as a function of age [13].

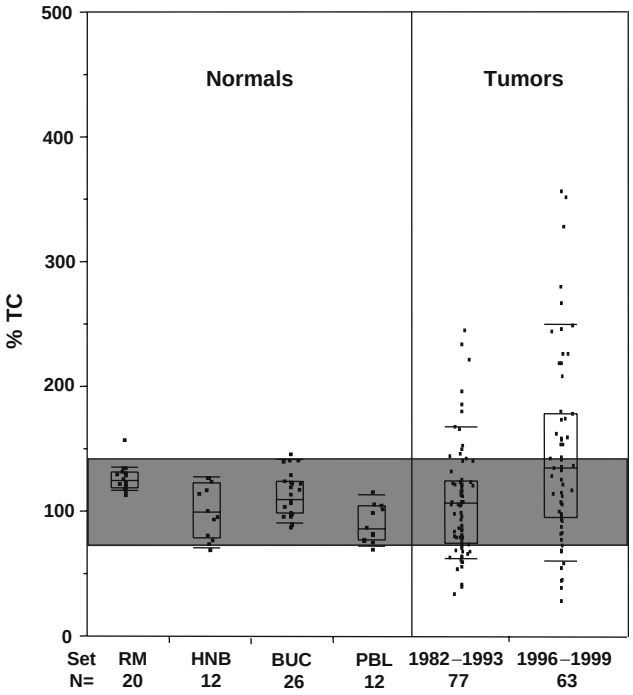

Figure 1. Distributions of telomere DNA contents (TC) in normal and tumor tissues. TC is shown on the *y*-axis, and is expressed as a percentage of TC in placental DNA standard, measured in parallel. The number of specimens in each tissue set (*N*) is indicated below the set designation on the *x*-axis. The shaded area (75–143% of the placental DNA standard) contains 95% of the TC values in the four sets of normal tissues. The line across the middle of each box shows the group median and the quartiles (25th and 75th percentiles) as its ends. The 10th and 90th quantiles are shown as lines above and below the box.

The inter-quartile range (IQR), a statistical measure of the dispersion of the TC data, was 28% for the combined normal tissues (Table 2). Ninety-five percent of all normal specimens had TC values of 75–143% of the standard (shaded area, Figure 1). In order to assess the extent to which this range was truly representative of normal tissues, we measured TC in a second, independent collection of 60 normal tissues (9 renal, 1 bone marrow, 2 breast, 2 lymph node, 2 prostate, 1 tonsil and 43 PBL). Similarly, 95% of the specimens had TC values within 75–145% of the standard (data not shown). Therefore, the distributions of TC in normal tissues is approximately 75–145%, which includes the effects of all extraneous factors, such as experimental variation, and inherent factors, such as subject's age and health status, the tissue type and source.

Telomere contents in breast tumor tissues differ from normal tissues

Matched tumor tissue was available for the 12 specimens of HNB tissues described above. Although TC in 11/12 of the HNB tissues fell within the expected range for normal tissues, only 2/12 matched tumors had TC within this range (Table 3). On average, TC in tumors was 61% of TC in the matched HNB tissues. TC was measured next in the 140 tumors comprising the 1982–1993 and 1996–1999 tumor sets (Figure 1). The IQR for TC in the two sets of tumor tissues, 49 and 79%, respectively, were substantially greater than the 28% IQR of the normal tissues (Table 2). Fifty-six percent of breast cancer specimens in the 1982–1993 set had TC values within the range that contained 95% of normal tissues, while 23 and 21% had TC values less and greater than the normal range, respectively. Similarly, only 43% of breast cancer specimens in the 1996–1999 set had TC values within the range that contained 95% of normal tissues, while 14% were below the range and 43% were above. Thus, TC in breast cancer tissues is significantly more heterogeneous than that in normal tissues, reflecting frequent abnormally short and long telomeres.

Table 3. TC in paired HNB and tumor tissue

Subject[a]	Telomere DNA content (% placental DNA control)		
	HNB (%)	Tumor (%)	T/N (%)
A	95	58	61
B	75	49	65
C	78	70	90
D	102	56	55
E	115	24	21
F	70	65	93
G	128	56	44
H	97	85	88
I	82	63	77
J	118	40	34
K	128	29	23
L	125	108	86
Average	101	59	61

Additional details are found in the text and the legend to Figure 1. T/N is the percent TC in the tumor (T) relative to TC in the paired, histologically normal (HNB) tissues.
[a]Tissue sets are described in the Materials and methods section.

Telomere contents in breast tumor tissues are associated with TNM stage

As shown in Table 2, mean and median TC differed between 1982–1993 and 1996–1999 tumor sets. A non-parametric Rank–Sums test of this difference in the means (109 and 148%, respectively) was highly significant ($p = 0.0008$). There were also highly significant differences between the two sets in the women's ages at diagnosis ($p = 0.001$), and their tumor's sizes ($p < 0.0001$), nodal involvements ($p = 0.0009$) and TNM stages ($p < 0.0001$). In order to more directly address a possible relationship between TC and the age at diagnosis, tumor size, nodal involvement and TNM stage, the two tumor sets were combined and these relationships were evaluated by non-parametric Rank–Sums tests (Figure 2a–c) and logistic regressions (Figure 2f–h). In each instance, there were highly significant associations with TC. Approximately 85% of the tumors in the 1982–1993 set were TNM stage IIA or higher; while approximately 66% of tumors in the 1996–1999 set were TNM stage 0 or I (Table 1). This, coupled with the strong association between TC and node status, suggests that TC discriminates across TNM stages. In contrast, there was no detectable association between TC and tumor histology (i.e. ductal versus lobular carcinomas).

Telomere contents in breast tumor tissues are associated with breast cancer survival

We hypothesized that telomere DNA length in a tumor is a surrogate for phenotypic variability and, therefore, atypically long and short telomeres, measured by high and low TC, respectively, are more likely associated with favorable and poor clinical outcomes, respectively. At least 5 years of follow-up data were available for 137 of the 140 women in the 1982–1993 and 1996–1999 sets. The relationships between TC and both overall 5-year survival and breast cancer-free 5-year survival were evaluated by non-parametric Rank–Sums tests (Figure 2d,e) and logistic regressions (Figure 2i,j). Both methods demonstrated highly significant associations between TC and overall 5-year survival ($p = 0.0001$, $p < 0.0001$, respectively) and breast cancer-free 5-year survival ($p = 0.0004$, $p = 0.0002$, respectively). The same conclusion was reached when the two tumor sets were analyzed separately (data not shown). In these analyses, the Kruskal–Wallis tests demonstrated that TC in the 1982–1993 group was associated with both overall 5-year survival ($p = 0.01$) and breast cancer-free 5-year survival ($p = 0.005$). TC in the 1996–1999 set was also associated with overall 5-year survival ($p = 0.02$)

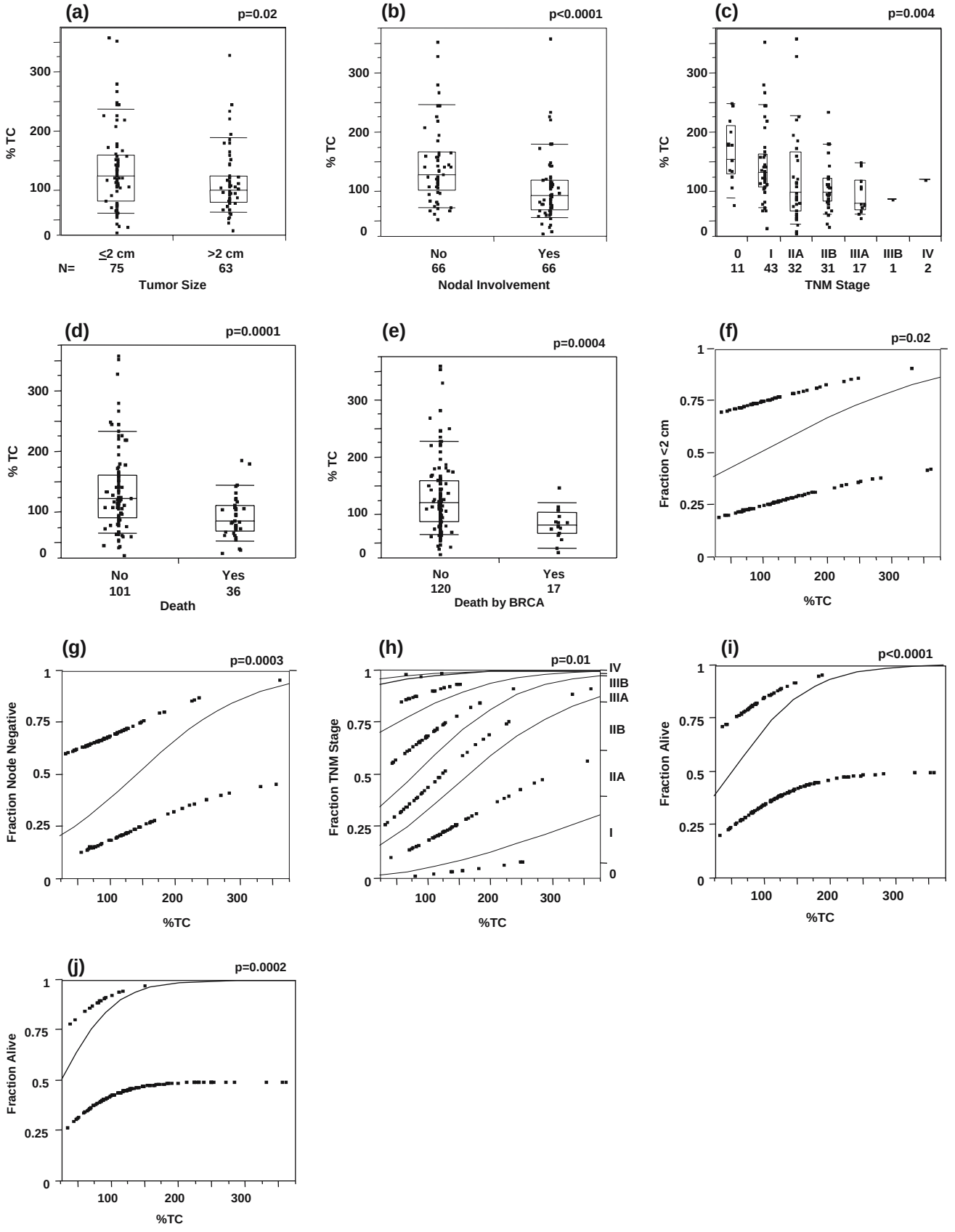
(a)
p=0.02
% TC
300
200
100
0
≤2 cm
>2 cm
N= 75 63
Tumor Size
(b)
p<0.0001
% TC
300
200
100
0
No
Yes
66 66
Nodal Involvement
(c)
p=0.004
% TC
300
200
100
0
0 I IIA IIB IIIA IIIB IV
11 43 32 31 17 1 2
TNM Stage
(d)
p=0.0001
% TC
300
200
100
0
No
Yes
101 36
Death
(e)
p=0.0004
% TC
300
200
100
0
No
Yes
120 17
Death by BRCA
(f)
p=0.02
Fraction <2 cm
1
0.75
0.5
0.25
0
100 200 300
%TC
(g)
p=0.0003
Fraction Node Negative
1
0.75
0.5
0.25
0
100 200 300
%TC
(h)
p=0.01
Fraction TNM Stage
1
0.75
0.5
0.25
0
IV
IIIB
IIIA
IIB
IIA
I
0
100 200 300
%TC
(i)
p<0.0001
Fraction Alive
1
0.75
0.5
0.25
0
100 200 300
%TC
(j)
p=0.0002
Fraction Alive
1
0.75
0.5
0.25
0
100 200 300
%TC

Figure 2. Associations between breast tumors' telomere DNA contents (TC) and tumor size, nodal status, TNM stage and 5 year breast cancer-free survival. Tumor sets 1982–1993 and 1996–1999 were combined and stratified by tumor size (a), nodal status (b), TNM stage (c), overall 5-year survival (d) and breast cancer-free 5-year survival (e). TC is shown on the *y*-axis, and is expressed as a percentage of TC in placental DNA standard, measured in parallel. The number of specimens in each tissue set (*N*) is indicated below the set designation on the *x*-axis. Statistical significance (*p*) was determined using the 2-sided non-parametric Rank–Sums test. The relationships between TC and tumor size (f), nodal status (g), TNM stage (h) overall 5-year survival (i) and breast cancer-free 5-year survival (j) were also evaluated by logistic regression. Logistic regression estimates the probability of choosing one of the specified parameters (e.g. large vs. small tumors) as a continuous function of TC. In a logistic probability plot, the *y*-axis represents probability. TC is shown on the *x*-axis, and is expressed as a percentage of TC in the placental DNA standard. The proportion of small tumors (i.e. <2.0 cm), node negative tumors, TNM stage 0–IV tumors, and survivors are shown on the *y*-axis. See the legend to Figure 1 for additional details.

however, no members of the 1996–1999 set died from breast cancer within 5 years of surgery (Table 1). Highly significant relationships between TC and overall 5-year survival ($p = 0.01$) and breast cancer-free 5-year survival ($p = 0.002$) in the 1982–1993 group, and overall 5-year survival in the 1996–1999 set ($p = 0.02$) were also detected by logistic regression. Collectively, the data support the conclusion that longer telomeres are protective while shorter telomeres presage poor survival.

The sensitivity and specificity of TC as a predictor of breast cancer-related death was evaluated by analysis of the TC's receiver operating characteristics (Figure 3). TC ranges for the lower, middle and upper tertiles in normal tissues were <101, 101–123, and >123% of standard, respectively. Consistent with the data in Figure 1 demonstrating that many tumors have TC values that are greater or lesser than those typically observed in normal tissues, only 20 and 14% of tumors in the 1982–1993 and 1996–1999 sets, respectively, had TC values within the range defined by the middle tertile. The 124% cutoff predicted 5 year survival with approximately 50% sensitivity and 95% specificity, while the 100% TC cutoff predicted death due to breast cancer with approximately 75% sensitivity and 70% specificity.

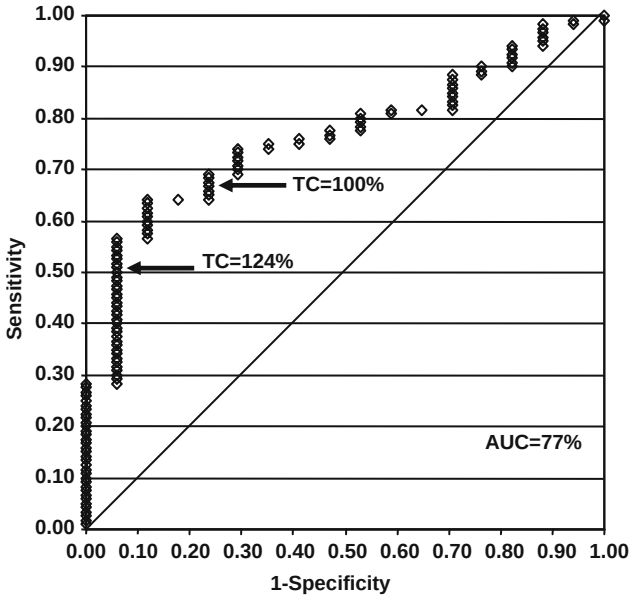

Figure 3. Receiver operating characteristics (ROC) analysis of the relationship between TC in breast tumors and 5 year survival. The specificity and sensitivity of TC as a predictor of 5 year survival following diagnosis of breast cancer was calculated for each value of TC in the combined 1982–1993 and 1996–1999 sets. Seventeen subjects died from breast cancer, and 119 survived for at least 5 years after diagnosis. The plot shows 1-specificity (the false positive rate) on the *x*-axis and sensitivity (1 – the false negative rate) on the *y*-axis. Arrows correspond to the high and low TC cutoffs (100 and 124%) that define the boundaries of the lower and upper tertiles of TC in normal tissues. See text for additional details. The area under the curve (AUC) is 77%.

Telomere contents in breast tumor tissues predict breast cancer-free survival interval

The extensive follow up data associated with the 77 tumors in 1982–1993 set (up to 23 years) made it possible to evaluate the effect of TC on breast cancer-free survival. The tumors were grouped using the TC thresholds described above: low TC was defined as less than or equal to 100%, intermediate TC was defined as 101–123%, and high TC was defined as greater than 123%. A Kaplan–Meier plot and Log–Rank test (Figure 4) demonstrated significant differences in the groups' survival intervals ($p = 0.013$). This effect is independent of age at diagnosis, nodal involvement and TNM stage (Table 4).

As shown in Table 5, low TC conferred an unadjusted relative hazard of 4.39 (95% CI = 1.47–13.08;

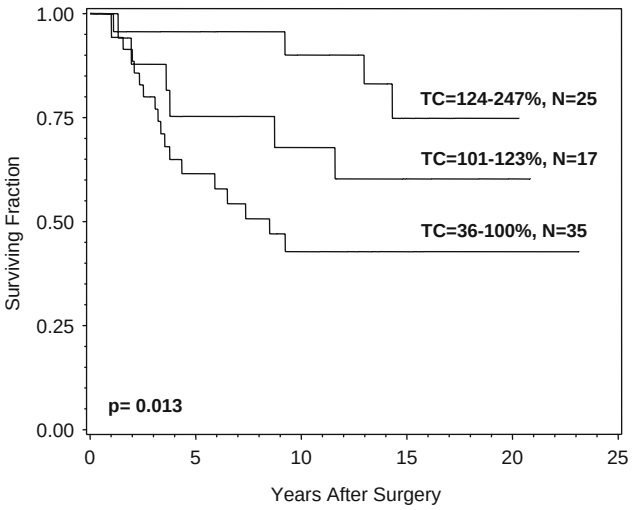

Figure 4. Breast cancer death by telomere DNA content in breast tumors. The 1982–1993 set was divided into three groups based on the high and low TC cutoffs (100 and 124%) that define the boundaries of the lower and upper tertiles of TC in normal tissues. Breast cancer-free survival interval, in years, is shown on the *x*-axis and the recurrence-free fraction is shown on the *y*-axis. The Log–Rank test was used to test the significance of the differences in the groups survival intervals ($p = 0.013$). *N* represents the number of subjects in each group. See Materials and methods section for additional details.

Table 4. TNM stage, lymph node involvement, mean age at diagnosis and tumor size by TC level

	TC level					
	36–100%		101–123%		124–247%	
	N	%	*N*	%	*N*	%
TNM stage						
I	3	8.6	3	17.6	5	20.0
IIA	11	31.4	2	11.8	7	28.0
IIB	12	34.3	6	35.3	7	28.0
IIIA, IIIB, IV	9	25.7	5	29.4	5	20.0
Unknown	0	0.0	1	5.9	1	4.0
Lymph nodes						
Negative	8	22.9	5	29.4	12	48.0
Positive	27	77.1	12	70.6	12	48.0
Unknown	0	0.0	0	0.0	1	4.0
	N	Mean	*N*	Mean	*N*	Mean
Age at diagnosis	35	56.3	17	46.9	25	48.5
Tumor Size (mm)[a]	35	36.1	15	32.1	25	32.1

Abbreviations: TC, telomere DNA content; *N*, number of specimens.
[a]Size is measured in longest dimension.

Table 5. Relative hazards and 95% confidence intervals from proportional hazards model of survival from date of diagnosis of breast cancer

	Unadjusted		Adjusted for age, TNM stage	
	RH (95% CI)	*p*-Value	RH (95% CI)	*p*-Value
TC level				
36–100	4.39 (1.47, 13.08)	0.0079	4.43 (1.44, 13.64)	0.0094
101–123	2.33 (0.66, 8.27)	0.1900	1.95 (1.54, 7.06)	0.3066
124–247	1.00		1.00	

A proportional hazards model of survival from date of diagnosis of breast cancer and up to 23 years of follow up was used to derive the unadjusted and adjusted relative hazards (RH) associated with each TC group. The adjusted RH was developed using age at diagnosis, TNM Stage and TC as independent predictors of survival. The 95% confidence intervals for RH are shown in parenthesis. Abbreviations: TC, telomere DNA content; RH, relative hazard; CI, confidence interval. See Materials and methods section for additional details.

$p = 0.008$) relative to high TC. A multivariable Cox model for the 1982–1993 breast tumor tissue set was developed using age at diagnosis, TNM stage and TC as independent predictors of breast cancer-free survival. Relative to the high TC group, low TC conferred an *adjusted* relative hazard of 4.43 (95% CI = 1.44–13.64; $p = 0.009$). In total these data demonstrate that TC predicts clinical outcome in invasive breast cancer.

Discussion

Telomere DNA content (TC) is a convenient proxy for telomere length that is particularly well-suited for the analysis of samples where DNA is degraded or scant, such as sections from archival, paraffin-embedded tissues. We measured TC values in three independent sets of cancerous breast tissues, compared these to TC in four sets of normal breast, buccal and blood cells, and evaluated the associations of TC with tumor markers and clinical endpoints, including disease-free and overall survival, in two independent cohorts comprising a total of 140 women with invasive breast cancer.

Four principal findings were made from this study. The first is that the range of telomere lengths in each of the three sets of breast tumors, measured as TC, is significantly greater than the range of TC in tissues from disease-free breast, buccal cells and blood cells. Only 17% of all tumors had TC values that were within the range defining the middle tertile of normal tissues, and approximately half of all tumors had TC values greater or lesser than those in 95% of normal tissues. These differences exceed those attributable to the several inherent and extraneous factors that can potentially confound measurements of telomere length, including age, and demonstrate the disparity between the regulation of telomere length in normal and tumor cells. It is significant that TC was associated with age in normal tissues, but not in tumors. This suggests that the extent of telomere attrition and the activities of the compensatory mechanisms that lengthen and stabilize telomeres, such as telomerase-dependant or -independent (“ALT”) processes, occurring in tumor cells are sufficiently large to obscure the underlying, age-dependent differences in telomere length.

Second, TC had significant associations with TNM stage (0 or I versus IIA and higher) and also two of its components: tumor size and nodal status. In contrast to previous studies, and our investigation of prostate tumors, where TC cutoffs were defined arbitrarily [20], TC cutoffs in the present study were derived from the distribution of TC values *in normal tissues*. Given the small amounts of DNA necessary to measure TC (as little as 5 ng), these results suggest that TC obtained by needle biopsy or fine needle aspirates (FNA) may be used to provide physicians preliminary TNM staging (or nodal involvement) information prior to surgery.

We next demonstrated an association between TC in breast tumor DNA and vital status following surgery. Even though the two tumor sets were not controlled for adjuvant therapies, the relationships between TC and overall 5-year survival and breast cancer-free 5-year survival were highly significant ($p = 0.0001$ and $p =$ c0.0004, respectively). TC thresholds based on the tertile distributions in normal tissues (described above) predicted 5 year breast cancer-free survival with approximately 50% sensitivity and 95% specificity and death resulting from breast cancer within 5 years of surgery with approximately 75% sensitivity and 70% specificity. Kaplan–Meier plots confirmed that TC was associated with the breast cancer-free interval.

Finally, TC provides prognostic information that is independent of its ability to discriminate disease stage. The relative hazard for death by breast cancer following diagnosis that is conferred by TC values $\leq 100\%$, after controlling for age at diagnosis and TNM stage involvement (RH = 4.43), was highly significant ($p =$ 0.009). This result is nearly identical to our prior finding that the relative hazard for recurrence of prostate cancer following prostatectomy conferred by TC values $\leq 75\%$, after controlling for age at diagnosis, Gleason sum, and pelvic node involvement (RH = 5.02) was also significant ($p = 0.013$) [20]. Together, these data support the hypothesis that TC provides *independent* prognostic information in multiple solid tumor types. We hypothesize that telomere content predicts the likelihood of micrometastasis and, in combination with extant prognostic markers, might have better predictive value than the extant markers alone, thus providing patients and their physicians new information to guide therapeutic decisions.

It is important to point out that all of the analyses reported herein were performed with DNA purified from tumor tissues that had *not* been microdissected. Although histological review of tissue sections indicated that tumor cells typically comprised 75–100% of the samples, the potentially confounding effects of contaminating normal cells in the tumor warrants consideration. In this context, we recently demonstrated that telomere attrition *comparable to that in matched prostate and breast tumor tissues* occurs in histologically normal tissues at distances at least one centimeter from the visible tumor margins [20,31]. In the latter study, it was estimated that at least 40% of the cells in the tumor adjacent histologically normal (TAHN) breast tissues were genetically aberrant, and more than a third of unbalanced alleles in the tumor were conserved in matched TAHN breast tissues, implying that the tumor and TAHN cells were derived from the same progenitor. Taken together, these data support the conclusion that TC in tumors and ''contaminating'' normal cells are comparable, thus precluding the requirement for tissue microdissection.

In summary, we report consistent differences in TC between normal, disease-free and cancerous breast tissues that are statistically significant by tumor characteristics and clinical outcome. We conclude that TC is a marker associated with disease stage and, importantly, appears to be an independent predictor of clinical outcome and survival.

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Telomere DNA Content Predicts Breast Cancer–Free Survival Interval

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Abstract

Background: Telomeres are nucleoprotein complexes that protect chromosome ends from degradation and recombination. Critically shortened telomeres generate genomic instability. It has been postulated that the extent of telomere DNA loss is related to the degree of genomic instability within a tumor and therefore may presage clinical outcome. The objective of this investigation was to evaluate the hypothesis that telomere DNA content (TC) in breast tumor tissues predicts breast cancer–free survival interval.

Materials and Methods: Slot blot titration assay was used to quantitate TC in 530 archival breast tumor tissues in a population-based cohort. The relationships between TC, 12 risk factors for breast cancer adverse events (i.e., death due to breast cancer, breast cancer recurrence, or development of a new primary breast tumor), and breast cancer–free survival interval were evaluated by Fisher's exact test, log-rank analysis, and univariate and multivariate Cox proportional hazards models.

Results: TC was independent of each of the 12 risk factors. Ethnicity, tumor-node-metastasis stage, estrogen receptor, progesterone receptor, and p53 status, chemotherapy sequence, adjuvant therapy, and TC each conferred significant relative hazards. The best overall multivariate Cox proportional hazards model included TC, p53 status, tumor-node-metastasis stage, and estrogen receptor status as independent predictors of breast cancer–free survival interval ($P < 0.00005$). Low TC (≤200% of standard), relative to the high-TC group (>200% of standard), conferred an adjusted relative hazard of 2.88 (95% confidence interval, 1.16-7.15; $P = 0.022$) for breast cancer–related adverse events.

Conclusions: TC in breast cancer tissue is an independent predictor in this group of breast cancer–free survival interval.

Therapeutic management of breast cancer is complicated by the reality that conventional prognostic markers, such as patient age, tumor-node-metastasis (TNM) stage, and hormone receptor status, often do not identify women who will have a local or distant recurrence (1–3). Hence, many women are unintentionally overtreated or undertreated. For example, approximately one-third of women with breast cancer are node-negative at the time of diagnosis, of whom ~80% and 70% will survive for 5 and 10 years, respectively, if treated with surgery and radiotherapy alone (1). Adjuvant polychemotherapy in node-negative patients with ages <50 years improves 10-year survival from 71% to 78%, whereas in patients with ages 50 to 70 years, adjuvant therapy improves 10-year survival from 67% to only 69% (1). However, because currently available staging and prognostic markers cannot reliably identify the minority of women who will benefit from adjuvant therapy, the NIH/National Cancer Institute and St. Gallen guidelines each recommend adjuvant polychemotherapy for all women with moderate-risk to high-risk breast cancer (2, 3). Consequently, the majority of women with localized tumors have therapy-related side effects and reduced quality of life while gaining no therapeutic benefit (4). Thus, there is a pressing need for new markers that accurately predict the likelihood of breast cancer recurrence.

Tumorigenesis in humans is a multistep process in which successive genetic alterations, each conferring a selective advantage, drives the progressive transformation of normal cells into highly malignant cancer cells (5). Due to incomplete replication, telomeres, the nucleoprotein complexes that protect the ends of eukaryotic chromosomes from degradation and recombination, are shortened during each round of cellular

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replication (6), resulting in a reduction in telomere length with each cycle of chromosome replication (7, 8). Consequently, there is a limit to the number of doublings somatic cells can undergo before telomeres are critically shortened, become dysfunctional, and trigger successive rounds of chromosome breakage-bridge-fusion cycles, thus driving chromosome amplification, loss or structural rearrangement, and, consequently, tumorigenesis (5, 9–12).

The relationship between dysfunctional telomeres, genomic instability, and altered gene expression implies that tumors with the shortest telomeres have the most unstable genomes and, consequently, the greatest probability of aberrant gene expression. Likewise, tumors with the longest telomeres would be expected to have fewer genomic alterations and, therefore, lower probability of containing cells with the phenotypes associated with disease recurrence. Accordingly, several recent studies suggest telomere length may provide independent prognostic information for several solid tumors, including breast cancers (reviewed in ref. 13). However, measurement of telomere length in formalin-fixed, paraffin-embedded (FFPE) tissues that are typically available for retrospective studies is problematic due to the limited quantity and poor quality of the DNA that is recovered. Methods that are not affected by these limitations, such as telomere fluorescence *in situ* hybridization, are not well suited for the high-throughput analyses needed for large sample sets (14).

To circumvent these problems, we previously described a method for measuring telomere length in genomic DNA obtained from fresh, frozen, and, most importantly, FFPE tissues (15, 16). The content of telomere DNA sequences (TC) in a DNA sample is titrated by hybridization on a slot blot with a telomere-specific probe and then normalized to the quantity of total genomic DNA in the same sample, thus controlling for the differences in DNA ploidy that are frequent in solid tumors. TC is particularly well-suited for use with DNA from archival tissues: TC is directly proportional to telomere length measured by Southern blot ($r = 0.904$), can be measured with as little as 5 ng of genomic DNA, is insensitive to fragmentation of the DNA to <1 kb in length, and can be measured successfully in DNA from FFPE tissues stored for up to 20 years at room temperature (15–18).

Using this method, we have recently shown that TC is associated with breast cancer–free survival interval [relative hazard, 4.43; 95% confidence interval (95% CI), 1.44-13.64; $P = 0.009$], controlling for age at diagnosis and TNM stage (17). This study and other investigations (reviewed in ref. 13) provide strong evidence that TC predicts clinical outcome. However, our previous study had a retrospective design (which is more open to bias than the current prospective study), included a limited number ($n = 77$) of specimens collected in the mid 1980s and early 1990s, and was not controlled for the effects of adjuvant treatments and other clinical and prognostic variables. Therefore, it is unknown how TC would perform as a prognostic marker in a contemporary, population-based cohort, in which most tumors are detected by screening at earlier stages and many women elect breast-sparing surgery with adjuvant radiation, chemotherapy, or hormonal therapy.

In the current investigation, we addressed these questions by assessing the relationship between TC and breast cancer–free survival interval in FFPE tumor specimens obtained from 530 members of the New Mexico subset of the National Cancer Institute/Surveillance, Epidemiology, and End Results Health, Eating, Activity and Lifestyle (HEAL) prospective, population-based cohort (19).

Materials and Methods

Tissue samples. The HEAL study is an ongoing population-based, multicenter prospective cohort study of women diagnosed with breast cancer designed to evaluate the association between body composition, hormones, diet, physical activity, and prognosis over time for non-Hispanic White, Hispanic, and African-American women ascertained through the Surveillance, Epidemiology, and End Results registries[3] (19). In New Mexico, incident cases were ascertained by the New Mexico Tumor Registry. Eligibility was based on a first primary breast cancer diagnosis with *in situ* or stages I to IIIA breast cancer (based on the revised 2002 American Joint Committee on Cancer stage groupings; ref. 20) between July 1, 1996 and March 31, 1999, with ages 18 years or more, and residence in one of five centrally located New Mexico counties (Bernalillo, Santa Fe, Sandoval, Valencia, Taos). Women completed a postdiagnosis interview, blood draw, and anthropometric measurements. A total of 998 eligible first primary breast cancer cases were ascertained. Of the eligible cases, 615 patients (61%) chose to participate in the study. Participation rates were 55% for Hispanics and 64% for non-Hispanic Whites. Reasons for nonparticipation or exclusion included physician refusal (3%), unable to locate or interview subject (12%), and subject refusal (24%). Of the 615 total eligible patients for the study, 530 cases (86%) had slides retrieved for subsequent TC analysis, and there was no statistically significant difference in the block retrieval rates between cases with invasive and *in situ* disease. Lymph node status, tumor size, age, chemotherapy, adjuvant therapy, hormonal therapy, and menopausal status were based on medical record abstraction. Lymph node status was based on whether nodes were examined, and the number was identified as positive or negative for cancer. Ethnicity and family history were based on self-report at the time of interview. Coded data, stripped of all personal identifiers (Table 1), were provided by the HEAL investigators (R.N.B. and K.B.B.) and the New Mexico Tumor Registry, as approved by the University of New Mexico Human Research Review Committee. The mean age and follow-up of cohort members were 59.1 (range, 29-89; SD, 12.5) and 6.7 (range, 0.45-9.16; SD, 1.6) years, respectively. At the time of analysis, 83% of the cohort members were alive. Additionally, 85% of the cohort members were free of disease, either at time of analysis or at time of their non–breast cancer–related deaths.

Histologic review. FFPE tissue sections were obtained from the original diagnostic material, stained with H&E and examined microscopically by a surgical breast pathologist. Tissue sections were not microdissected and typically contained from 75% to 100% tumor cells.

Determination of TC. DNA was extracted from four 10-μm FFPE tissue sections, and TC was measured in known masses, typically 5 to 10 ng, by slot blot titration assay, as previously described (17, 18). TC is expressed as a percentage of the TC in a placental DNA standard measured in parallel. Each measurement was repeated independently thrice and the coefficient of variation for each sample was <10%.

Immunohistochemistry. Immunohistochemistry was done on FFPE breast tumor sections to determine hormone receptor, p53, and HER2/*neu* status. Hormone receptor assays were conducted in laboratories associated with the hospitals, wherein cases were diagnosed. p53 protein expression was evaluated using the anti-p53 monoclonal antibody DO-7 (Santa Cruz Biotechnology), which recognizes both the mutated and wild-type protein (21). p53 tumor suppressor gene mutations occur in 20% to 50% of breast carcinomas (22) and have been reported to be associated with poor prognosis (23). Mutations in p53 are predominantly missense and lead to conformational alterations

[3] http://appliedresearch.cancer.gov/surveys/heal/

Table 1. Relative hazards of risk factors for breast cancer–related adverse events in the HEAL patient cohort by TC level

Characteristic	All patients				High TC		Low TC	
	n	Percentage (*n* = 530)	Relative hazard (95% CI)	*P*	*n*	Percentage (*n* = 86)	*n*	Percentage (*n* = 444)
Ethnicity								
Non-Hispanic White	408	77	1.0		69	80	338	76
Hispanic	122	23	1.78 (1.11-2.84)	0.017	17	20	106	24
TNM stage								
0 (*in situ*)	97	18	1.0		17	20	80	18
I	259	49	0.92 (0.46-1.86)	0.820	43	50	216	49
IIA	115	22	1.87 (0.91-3.85)	0.087	19	22	96	22
IIB	41	8	3.73 (1.71-8.13)	0.001	5	6	36	8
IIIA	5	1	1.94 (0.25-15.02)	0.527	0	0	5	1
Tumor grade								
I	108	20	1.0		18	21	90	20
II	139	26	0.73 (0.36-1.48)	0.382	25	29	114	26
III	104	20	1.21 (0.62-2.37)	0.578	14	16	90	17
Estrogen receptor status								
Positive	444	84	1.0		71	83	373	84
Negative	82	15	2.62 (1.62-4.24)	0.0001	15	17	67	15
Progesterone receptor status								
Positive	359	68	1.0		62	72	297	69
Negative	168	32	2.04 (1.31-3.18)	0.002	23	28	144	32
p53 status								
Negative	262	49	1.0		44	51	218	49
Focal	151	28	0.99 (0.56-1.73)	0.966	19	22	132	30
Low	28	5	1.04 (0.37-2.94)	0.938	8	9	20	5
High	71	13	2.48 (1.44-4.27)	0.001	12	14	59	13
Age at Diagnosis								
<55	232	44	1.0		43	50	189	43
>55	298	56	0.76 (0.49-1.18)	0.220	43	50	254	57
Family history								
None	244	46	1.0		41	48	203	46
1° relative	128	24	0.87 (0.49-1.54)	0.627	20	23	108	24
2° relative	108	20	1.05 (0.59-1.86)	0.873	17	20	91	20
HER2/*neu* status								
Negative	300	57	1.0		49	57	251	57
Focal	111	21	0.95 (0.54-1.67)	0.845	16	19	95	21
Low	63	12	0.81 (0.38-1.71)	0.576	10	13	53	12
High	50	9	1.19 (0.58-2.44)	0.629	9	10	41	9
Chemotherapy								
None	406	77	1.0		71	83	335	75
After surgery	118	22	1.91 (1.20-3.05)	0.007	15	17	103	23
Adjuvant therapy								
None	178	33	1.0		26	30	152	34
Radiation	220	42	1.11 (0.63-1.98)	0.713	44	51	176	40
Chemotherapy	30	6	3.25 (1.52-6.95)	0.002	1	1	29	7
Both	102	19	1.83 (0.99-3.38)	0.052	15	17	87	20
Tamoxifen								
Yes	250	47	1.0		45	52	205	46
No	280	53	0.71 (0.45- 1.12)	0.143	41	48	239	54
Postmenopausal								
No	156	29	1.0		25	29	131	30
Yes	358	68	0.79 (0.49-1.26)	0.323	59	69	299	67
TC								
>200%	86	16	1.0		86	100	0	0
≤200%	444	84	3.14 (1.27-7.76)	0.013	0	0	444	100

NOTE: TNM stage was assigned using the 2002 revised criteria (20). Ethnicity and family history were based on self-report. See Materials and Methods for additional details.

of the protein and accumulation in tumor cell nuclei (24, 25). The cutoff levels for staining for p53 are negative (no staining), focal (<5% staining), low (5-39% staining), and high (40-100% staining). HER2/*neu* protein expression was evaluated using the anti-HER2/*neu* monoclonal antibody CB11 (Santa Cruz Biotechnology). The cutoff levels for staining for HER2/neu are negative (no staining observed or membrane staining observed in <10% of tumor cells), focal (faint/barely perceptible membrane staining detected in >10% of tumor cells and cells only stained in part of their membrane), low (weak to moderate complete membrane staining observed in >10% of tumor cells), and high (moderate to strong complete membrane staining observed in >10% of tumor cells). The negative and focal groups are

considered clinically negative; whereas, the low and high groups are considered clinically positive.

Statistical methods. The distribution of risk factors in the high-TC and low-TC groups (Table 1) was evaluated by the Fischer's exact test. Missing data for each risk factor was evaluated categorically in the analysis, but these data were not reported. The associations between TC and both overall survival interval and breast cancer–free survival interval were evaluated using log-rank Kaplan-Meier survival analyses. Univariate and multivariate Cox proportional hazards analysis was used to compute the relative hazards for breast cancer–related adverse events (i.e., death due to breast cancer, breast cancer recurrence, or development of a new primary breast tumor), and the best overall model, defined as the lowest overall model fit *P* value, is reported. Covariate-adjusted estimates of the survival function by level of TC (≤200% versus >200%) are the baseline survival estimates from a stratified proportional hazards model and were computed at the mean level of the covariates. Subjects were censored at the time lost to follow-up. *P* values of <0.05 were considered significant for all tests.

Results

TCs predict overall survival. To confirm prior associations observed between TC and overall survival interval, the cohort was initially divided into sixths, the survival interval for each group was calculated, and the results were evaluated for statistical significance by log-rank analysis. Groups with statistically indistinguishable survival intervals were combined, and the process was repeated until only groups with significantly different survival intervals remained. Using this process, the cohort was stratified into two TC groups: low TC was defined as ≤200% of the placental DNA control (n = 444), and high TC was defined as >200% of TC in the placental DNA control (n = 86). Log-rank analysis showed a significant relationship between TC group and overall survival interval (P = 0.025), with low TC predicting a shorter survival interval. The results are plotted by the method of Kaplan and Meier and shown in Fig. 1A. A univariate Cox proportional hazards model showed low TC had an unadjusted relative hazard of 2.25 (95% CI, 1.09-4.64; P = 0.029) relative to high TC (not shown). The relationship between TC group and overall survival interval in the subset of invasive tumors (i.e., without the 97 ductal carcinoma *in situ* cases) was also evaluated. In this subset, log-rank analysis also showed a significant relationship between TC group and overall survival interval (P = 0.046). The results are plotted by the method of Kaplan and Meier and shown in Fig. 1B. A univariate Cox proportional hazards model showed low TC had an unadjusted relative hazard of 2.06 (95% CI, 1.00-4.26; P = 0.05) relative to high TC (not shown).

TCs predict breast cancer–free survival. Next, we refined our criteria to evaluate the prognostic value of TC in predicting breast cancer–related, adverse event–free survival interval. An adverse event was defined as death due to breast cancer, breast cancer recurrence, or development of a new primary breast tumor. Seventy-nine breast cancer–related adverse events had occurred by the time of the analysis, including 46 deaths, 15 recurrences, and 18 new primary breast tumors. A Kaplan-Meier plot and log-rank test (Fig. 2A) showed significant differences in the groups' survival intervals (P = 0.009) with low TC, again predicting a shorter survival interval. A univariate Cox proportional hazards model showed low TC had an unadjusted relative hazard of 3.14 (95% CI, 1.27-7.76; P = 0.013) relative to high TC (Table 1). The relationship between TC group and breast cancer–free survival in the subset of invasive tumors was also evaluated. In this subset, log-rank analysis also showed a significant relationship between TC group and breast cancer–free survival interval (P = 0.032). The results are plotted by the method of Kaplan and Meier and shown in Fig. 2B. A univariate Cox proportional hazards model showed low TC had

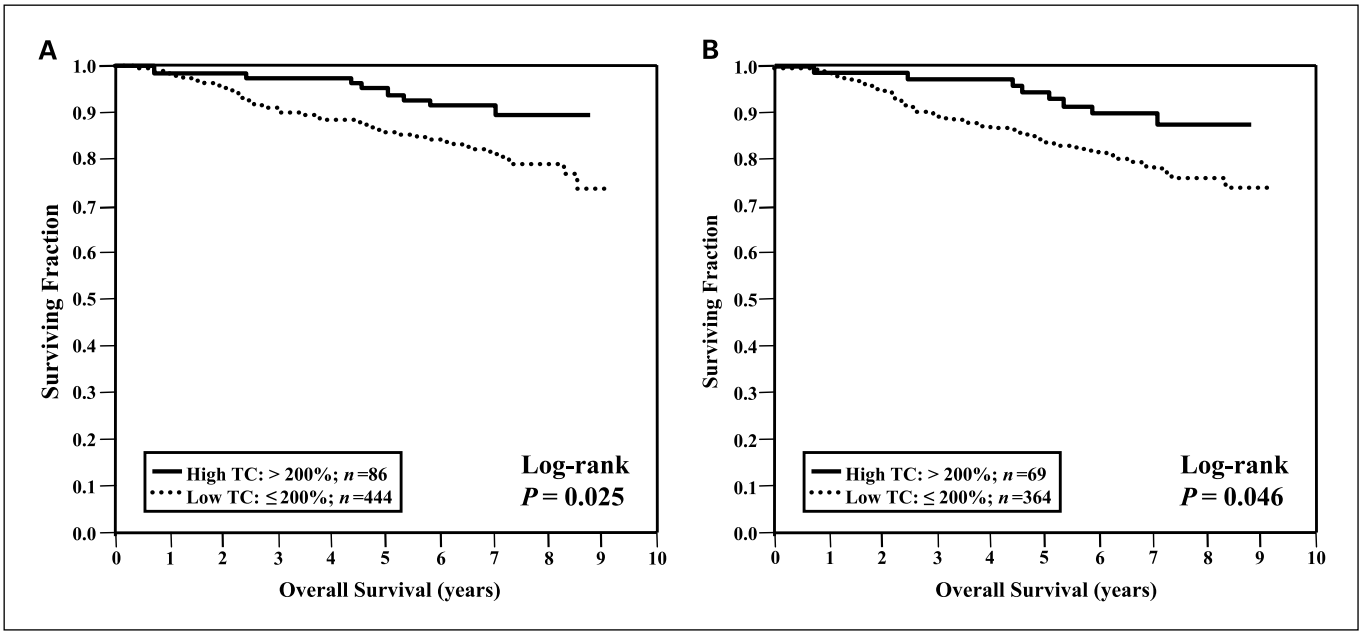

Fig. 1. Overall survival interval by TC in breast tumors. The set of all tumors (*A*) or invasive tumors only (*B*) was divided into two groups based on the low-TC and high-TC cutoff (200% of standard). Overall survival interval (in y) is shown on the *x* axis, and the surviving fraction is shown on the *y* axis. Subjects were censored at the time lost to follow-up. The log-rank test was used to test the significance (*P*) of the differences in the group's survival intervals. *n,* number of subjects in each group.

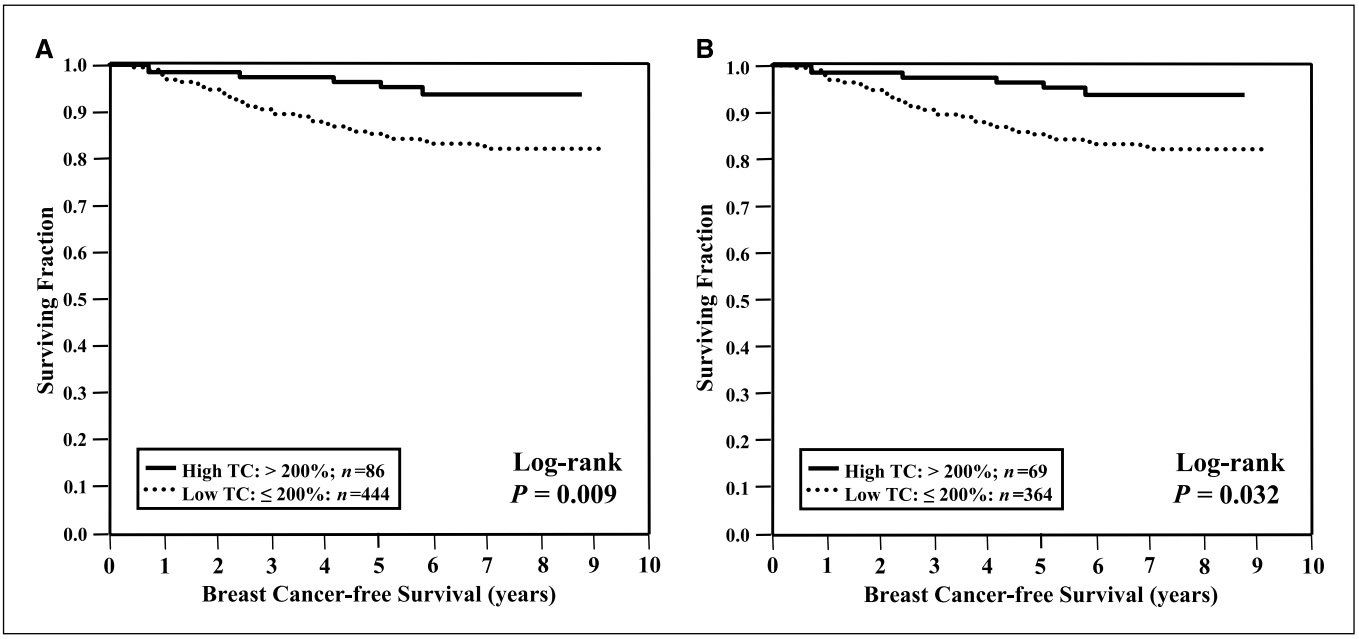

Fig. 2. Breast cancer – free survival interval by TC in breast tumors. The set of all tumors (*A*) or invasive tumors only (*B*) was divided into two groups based on the low-TC and high-TC cutoff (200% of standard). Breast cancer – free survival interval (in y) is shown on the *x* axis, and the recurrence-free fraction is shown on the *y* axis. See Fig. 1 for additional details.

an unadjusted relative hazard of 2.61 (95% CI, 1.05-6.48; $P = 0.039$) relative to high TC (not shown). Similarly, although not statistically significant, results were shown in the subset of ductal carcinoma *in situ* cases (not shown).

TC is an independent predictor of breast cancer–free survival. The relative hazards for breast cancer–related adverse events associated with 12 categorical risk factors were evaluated individually by Cox proportional hazards analysis (Table 1). Ethnicity, TNM stage, estrogen receptor, progesterone receptor, and p53 status, chemotherapy sequence, adjuvant therapy, and TC each conferred significant ($P < 0.05$) relative hazards. There was no significant hazard associated with age at diagnosis, family history of breast cancer, HER2/*neu*, or postmenopausal status or hormonal therapy. Pair-wise analysis using Fisher's exact test showed no significant difference in the distribution of any of the risk factors in the low-TC and high-TC groups (Table 1).

Multivariate Cox proportional hazards models were developed using TC and all combinations of the covariates that conferred significant relative hazards (ethnicity, TNM stage, estrogen receptor, progesterone receptor, and p53 status, chemotherapy sequence, adjuvant therapy, and TC). The best overall model (Table 2), defined as the lowest overall model fit *P* value, included TC, p53 and estrogen receptor status, and TNM stage ($P < 0.00005$). Relative to the high-TC group, low TC conferred an adjusted relative hazard of 2.88 (95% CI, 1.16-7.15; $P = 0.022$). The chemotherapy, adjuvant therapy, and hormonal therapy covariates were strongly associated with TNM stage and with each other ($P < 0.0001$). Therefore, additional multivariate Cox proportional hazards models were developed using TC and chemotherapy, adjuvant therapy, and hormonal therapy as covariates, either alone or in combinations. The best overall models, defined as the lowest overall model fit *P* value, included TC and either chemotherapy or adjuvant therapy ($P = 0.002$); the addition of the hormonal therapy covariate had no effect. In the second model, low TC conferred an adjusted relative hazard of 2.84 (95% CI, 1.14-7.05; $P = 0.025$), relative to the high-TC group (not shown).

Table 2. Relative hazards and 95% CIs from a multivariate Cox proportional hazards model of breast cancer–free survival interval from date of diagnosis of breast cancer

Variable	*n*	Level	Relative hazard (95% CI)	*P*
TC				
	86	>200%	1.00	
	444	≤200%	2.88 (1.16-7.15)	0.022
p53				
	441	None/focal/low	1.00	
	71	High	1.93 (1.10-3.38)	0.022
Estrogen receptor				
	444	Positive	1.00	
	82	Negative	1.69 (0.97-2.95)	0.063
TNM				
	259	I	1.00	
	97	0 (*in situ*)	0.98 (0.48-2.04)	0.967
	115	IIA	1.61 (0.90-2.88)	0.110
	46	IIB/IIIA	3.39 (1.81-6.36)	0.0001

NOTE: Multivariate Cox proportional hazards models of survival from date of diagnosis of breast cancer for breast cancer–free survival intervals were used to derive the adjusted relative hazards associated with each variable. Adjusted relative hazard values were developed using p53 status (none/focal/low versus high), TNM stage [I versus 0 (*in situ*) versus IIA versus IIB/IIIA], estrogen receptor status (present/absent), and TC group (≤200%/>200%) as independent predictors of survival. The 95% CIs for each relative hazard are shown in parenthesis. See Materials and Methods for additional details.

Discussion

TC is a convenient proxy for telomere length that is particularly well-suited for the analysis of samples where DNA is degraded or scant, such as sections from archival, FFPE tissues (15, 16). We used this method to determine TC values in tumor tissue collected in a prospective, population-based cohort composed of 530 women and evaluated the associations of TC with clinical variables and end points, including overall and breast cancer–free survival intervals.

The principal conclusion from this investigation is that TC predicts breast cancer–free survival interval, independent of 12 clinical factors, prognostic markers, and adjuvant therapies. Tumors with TC of ≤200% of placental DNA standard conferred an adjusted hazard for breast cancer recurrence of 2.88 (95% CI, 1.16-7.15; $P = 0.022$). These results, obtained from a large population-based cohort, are in accord with our recent study (17) of breast tumors (predominantly TNM stage IIA and above) that also showed highly significant associations between TC and overall 5-year survival ($P < 0.0001$) and breast cancer–free survival interval (relative hazard, 4.43; 95% CI, 1.44-13.64; $P = 0.009$). Likewise, our previous investigation of prostate cancer (18) revealed that TC was also associated with time to prostate cancer recurrence (relative hazard, 5.02; 95% CI, 1.40-17.96; $P = 0.013$), controlling for age at diagnosis, Gleason sum, and pelvic node involvement. Similar results were obtained when analyses were done using the subset of invasive tumors, and a similar trend was observed in the subset of ductal carcinoma *in situ* cases. These data suggest that TC may be able to predict clinical outcome in both invasive tumors and ductal carcinoma *in situ* cases. As discussed above, adjuvant polychemotherapy in node-negative patients with ages <50 years improves 10-year survival from 71% to 78% (a 24% increase, i.e., seven per 29%), whereas in patients with ages 50 to 70 years, adjuvant therapy improves 10-year survival from 67% to only 69% (a 6% increase, i.e., two per 33%). A TC threshold of >200% of the standard defines a subgroup comprising of ~17% of the population-based cohort that have a significantly reduced risk of disease recurrence (7% at 8 years) that would be potential candidates for less aggressive adjuvant therapy. However, subsequent experiments in larger cohorts are needed to extend these findings.

The point estimate of the relative hazard for breast cancer recurrence associated with "low" TC was lower than in our prior investigation (2.88 versus 4.43), although the confidence intervals overlap. One possibility is that the discrepancy in the point estimates reflects the difference in the length of follow-up in the two studies. The mean, maximum, and interquartile ranges for follow-up in the HEAL cohort were 6.7, 9.2, and 1.5 years, respectively, versus 9.1, 23, and 11.2 years, respectively, in the prior study (17). The ongoing follow-up of the HEAL cohort will resolve this question. It is also important to consider that HEAL is a prospective study in which FFPE tissue samples were collected for participants at multiple independent sites at the time of diagnosis before the start of follow-up, rather than a retrospective study of archival tissues from a single facility, which is more open to inadvertent selection bias.

Another important difference between these two studies is that the TC threshold used to discriminate women at risk for breast cancer recurrence, >123% and >200% in the prior and present studies, respectively. This difference may also reflect the differences in the lengths of follow-up, in which case we would expect that the threshold will decrease as more deaths and adverse effects occur. Alternatively, the discrepancies in threshold, as well as the point estimates for the relative hazard ratios, could reflect either the larger number of specimens (530 versus 77) or the larger fraction of localized tumors (stages 0 and I) in the HEAL cohort and prior cohort (67% versus 14%).

Here, using the HEAL cohort, we have shown that TC predicts breast cancer–free survival interval independent of other risk factors. It is important to note that these other established risk factors, such as ethnicity, TNM stage, estrogen receptor, progesterone receptor, and p53 status, chemotherapy, and adjuvant therapy also conferred significant univariate relative hazards for breast cancer–related adverse events, confirming a representative population cohort. However, this population was not selected for TC (or any other biomarker) analysis and, thus, represents an unbiased assessment of TC as a prognostic factor. Telomere shortening has been associated with age in normal tissues (26); however, in this study, there was no association between TC and patient age, which is consistent with our previous results (17, 18). This indicates that telomere attrition due to tumorigenesis far exceeds the shortening contributed to age alone. Additionally, it must be noted that the cutoff established in this study, >200% of the placental DNA standard, exceeds the 95% CI for TC in several normal tissues (75-143% of standard), including breast (17). Speculatively, these longer telomeres may result from the early up-regulation of telomerase during tumor progression.

In summary, TC in tissues from breast tumors is an independent predictor in this group of breast cancer–free survival interval. In the future, TC, in combination with extant prognostic markers, could provide women and their physicians new information to guide therapeutic decisions. However, the assay in its current format, due to the relatively complex experimental procedure, is more suitable for use in a research rather than clinical setting. Therefore, development of a platform for TC determination that is simple and readily adaptable to a clinical laboratory is necessary before these findings can be validated in independent laboratories with independent cohorts.

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PRECLINICAL STUDY

Genomic instability demonstrates similarity between DCIS and invasive carcinomas

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Abstract *Purpose* To assess telomere DNA content (TC) and the number of sites of allelic imbalance (AI) as a function of breast cancer progression. *Experimental design* TC and AI were determined in 54 histologically normal tissues, 10 atypical ductal hyperplasias (ADH), 122 in situ ductal carcinomas (DCIS) and 535 invasive carcinomas (Stage I–IIIA). *Results* TC was altered in ADH lesions (20%), DCIS specimens (53%) and invasive carcinomas (51%). The mean number of sites of AI was 0.26 in histologically normal group tissue, increased to 1.00 in ADH, 2.94 in DCIS, and 3.07 in invasive carcinomas. All groups were statistically different from the histologically normal group ($P < 0.001$ for each); however, there was no difference between DCIS and the invasive groups. *Conclusions* Genomic instability increases in ADH and plateaus in DCIS without further increase in the invasive carcinomas, supporting the notion that invasive carcinomas evolve from or in parallel with DCIS.

Keywords Allelic imbalance · Breast cancer · Ductal carcinoma in situ · Genomic instability · Telomere DNA content

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Introduction

It is widely accepted that genomic instability is a prerequisite for the initiation and progression of virtually all cancers [1]. Accordingly, the progression of breast cancer can be characterized by the accumulation of genetic mutations in critical genes accompanied by histological progression from normal epithelium to atypical ductal hyperplasia (ADH), to ductal carcinoma in situ (DCIS) to the development of an invasive breast carcinoma [2, 3].

A significant cause of genomic instability is telomere dysfunction [4–7]. Telomeres are nucleoprotein complexes that are comprised of 1,000–2,000 tandemly repeated copies of the hexanucleotide DNA sequence (TTAGGG) [8]. These repeat regions are associated with numerous telomere binding proteins, such as Telomeric Repeat-binding Factor 1 (TRF1), Telomeric Repeat-binding Factor 2 (TRF2) and Protection of Telomeres 1 (POT1), which play important roles in telomere maintenance [9, 10]. Telomeres are located at and stabilize the ends of eukaryotic chromosomes, thus preventing degradation and recombination [11–13]. However, telomeres can be critically shortened, and thereby become dysfunctional, by several mechanisms, including incomplete replication of the lagging strand during DNA

synthesis [14], loss or alterations of the telomere-binding proteins involved in telomere maintenance [15], and DNA damage induced by oxidative stress [16]. Telomere loss may be compensated by the reactivation of the enzyme telomerase, as seen in 85–90% of human cancers [17].

Abnormalities in telomere length are early and frequent events in the malignant transformation of numerous types of carcinomas [18, 19]. In breast, telomere shortening has been observed in invasive carcinomas, in situ lesions, and histologically normal tissue proximal to breast tumors [20, 21]. Additionally, our laboratory has recently demonstrated that telomere DNA content (TC), a proxy for telomere length, in breast tumor tissues is a prognostic marker for clinical outcome [22, 23].

Genomic instability can also be manifested by the presence of allelic imbalance (AI), which is a deviation from the normal 1:1 ratio of maternal and paternal alleles. Numerous studies have shown that the presence of AI is characteristic of invasive breast carcinomas [24, 25] and is also present at the in situ stage of the disease [26, 27]. Additional studies have demonstrated that AI occurs within atypical breast hyperplasias [28, 29], histologically normal tissue proximal to breast tumors [21, 30–32], and, in some instances, breast tissue from women with benign breast disease [33]. AI has also been found in the stromal compartment of cancer-associated breast tissues [34].

Numerous groups have investigated AI in the development of breast cancer. Notably, Ellsworth et al. [35] developed a panel of microsatellite markers specific for loci commonly lost in breast cancer. This group examined the evolution of genomic instability by characterizing AI in tissue samples representing a continuum of breast cancer development and concluded that DCIS lesions contain AI levels characteristic of advanced invasive tumors [36].

To evaluate the link between telomere dysfunction and the generation of allelic imbalance in the progression of breast cancer, we assessed alterations in TC and the extent of AI in a continuum of breast tissues ranging from histologically normal tissue derived from reduction mammoplasty, to ADH, DCIS and invasive carcinomas ranging from Stage I to IIIA. Here, we demonstrate that genomic instability (i.e. changes in TC or AI that exceed values typically observed in normal tissues) increases along the continuum of breast disease; however, it plateaus in DCIS without further increase in the invasive carcinomas.

Materials and methods

Tissue samples

A total of 721 human breast tissues were used in this study. Fifty-four normal, disease-free breast tissue samples from women undergoing reduction mammoplasty (mean age = 35.6 years; range: 17–68) were obtained from the National Cancer Institute Cooperative Human Tissue Network (Nashville, TN). Ten atypical ductal hyperplasia lesions (mean age = 56.3 years; range: 41–70) were obtained from the Department of Pathology at University of New Mexico Hospital (UNMH). Two independent cohorts of breast tumors were analyzed. The first cohort (test set) was obtained through the New Mexico Tumor Registry (NMTR) and Department of Pathology at UNMH and consisted of 163 specimens including DCIS ($N = 27$), and Stage I ($N = 104$) and IIA ($N = 32$) invasive breast carcinomas (mean age = 47.5 years; range: 25–77). The second cohort (validation set) was obtained through the Health, Eating, Activity and Lifestyle (HEAL) Study, an ongoing population-based, multi-center prospective cohort study [37], and consisted of 494 cases including DCIS ($N = 95$), and Stage I ($N = 244$), IIA ($N = 112$), Stage IIB ($N = 39$) and IIIA ($N = 4$) invasive breast carcinomas (mean age = 59.3 years; range: 29–89). Clinical data for the two tumor cohorts are shown in Table 1. Experiments were performed in accordance with all federal guidelines as approved by the University of New Mexico Health Science Center Human Research Review Committee.

Histological review

All tissue sections were examined microscopically to confirm diagnosis. Tissue sections were not microdissected, but typically contained from 75 to 100% tumor cells. A single pathologist reviewed the histological slides for the 10 ADH lesions and cohort two (validation set); whereas, the reduction mammoplasty specimens and cohort one (test set) were reviewed by numerous pathologists. The criteria used for the ADH specimens were based on morphological characteristics of a proliferative lesion that fulfills some but not all the criteria for DCIS.

DNA isolation and quantification

DNA was isolated from fresh, frozen or formalin-fixed, paraffin-embedded (FFPE) tissue samples using the DNeasy® silica-based spin column extraction kit (Qiagen; Valencia, CA) and the manufacturer's suggested animal tissue protocol. FFPE samples were treated with xylene and washed with ethanol prior to DNA extraction. DNA concentrations were measured using the Picogreen® dsDNA quantitation assay (Molecular Probes, Eugene, OR) using a λ phage DNA as the standard as directed by the manufacturer's protocol.

Table 1 Clinical and pathological characteristics of the analyzed breast tumors

Characteristic	Cohort #1 ($N = 163$)		Cohort #2 ($N = 494$)	
	N	%	*N*	%
Ethnicity				
NHW	106	65	380	77
Hispanic	27	17	114	23
Unknown	30	18	0	0
TNM stage				
0 (in situ)	27	16	95	19
I	104	64	244	49
IIA	32	20	112	23
IIB	0	0	39	8
IIIA	0	0	4	1
Node status				
Negative	163	100	261	53
Positive	0	0	107	22
Unknown	0	0	126	25
ER status				
Positive	80	49	418	85
Negative	46	28	72	14
Unknown	37	23	4	1
PR status				
Positive	72	44	340	69
Negative	53	33	151	30
Unknown	38	23	3	1
Age				
Mean	47.5		59.3	
Range	25–77		29–89	

TNM stage was assigned using the 2002 AJCC revised criteria. Ethnicity was self-reported. *N*, Number of specimens; ER, estrogen receptor; PR, progesterone receptor; NHW, non-Hispanic White. For additional details, see Materials and Methods Section

Telomere DNA content (TC) assay

TC was measured in known DNA masses, typically 5–10 ng, by slot blot titration assay, as previously described [21–23]. TC is expressed as a percentage of the TC in a placental DNA standard measured in parallel, which is defined as 100%. Each measurement was repeated independently three times and the coefficient of variation for each sample was $\leq$10%. The content of telomere DNA sequences can easily be measured in genomic DNA obtained from fresh, frozen and paraffin-embedded tissues [22, 38]. We have previously shown that TC is (i) directly proportional to telomere length determined by Southern blotting, (ii) not affected by TTAGGG sequences outside the telomere, and (iii) not affected by DNA fragmentation less than 1 KB in length [22, 38].

Determination of allelic imbalance

The extent of AI was determined using a straight-forward, economical, and high-throughput method recently developed by our laboratory [39]. This method evaluates AI in a panel of 16 randomly selected microsatellite markers (i.e. markers with no known relationship to breast cancer) thereby preventing measurement bias by selection of genes whose products are involved in tumorigenesis [39]. Briefly, DNA ($\sim$1 ng) was amplified using the AmpFlSTR Identifiler PCR Amplification Kit (Applied Biosystems, Foster City, CA) using the manufacturer's protocol. Each multiplex PCR reaction amplifies 16 short tandem repeat (STR) microsatellite loci from independent locations in the genome (Amelogenin, CSF1PO, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D19S433, D21S11, FGA, TH01, TPOX and vWA). Each of the PCR primers is labeled with one of four fluorescent dyes (6-FAM, PET, VIC and NED), each with a unique emission profile, allowing the simultaneous resolution of 16 amplicons of similar size. PCR products were resolved by capillary gel electrophoresis and detected using an ABI Prism 377 DNA Sequencer (Perkin Elmer, Foster City, CA). The height of each fluorescence peak in the electropherograms was quantitated using the ABI Prism GeneScan and Genotype Analysis software (Applied Biosystems, Foster City, CA) and a ratio of the peak heights of each pair of heterozygous allelic amplicons was calculated. By convention, the allele with the greater fluorescence intensity was designated the numerator. Thus, the ratio was always $\geq$1.0, with 1.0 representing the theoretical ratio for normal alleles. We previously defined an operational threshold of AI (i.e. $\geq$2 sites of AI) that could differentiate between a variety of normal and cancerous tissues independent of storage conditions (i.e. fresh, frozen or paraffin-embedded, formalin-fixed) [39]. Of the 118 normal specimens, only 1 (0.8%) specimen demonstrated $\geq$2 sites of AI. In contrast, of the 239 tumor specimens, 161 (67.4%) demonstrated $\geq$2 sites of AI.

Statistical methods

The mean number of sites of AI and TC distributions for histologically normal, ADH, DCIS and invasive carcinoma specimens were analyzed by non-parametric Rank Sums tests. Chi-square tests were used to determine differences for individual allelic frequencies between the DCIS and invasive groups. JMP® statistical package (SAS Institute, Cary, NC) was used for all analyses and P-values < 0.05 were considered to be significant.

Results

TC in histologically normal tissue and ADH lesions

TC was determined in 54 histologically normal breast tissues obtained from women who underwent reduction mammoplasty. TC was tightly regulated within these histologically normal breast tissues; 95% of these normal specimens fell within the range of 75–154% (Fig. 1), nearly identical to the 75–143% range previously reported in a diverse set of 70 specimens of normal tissue from multiple organ sites, including breast [22]. Next, TC was determined in a set of 10 ADH lesions. TC values in two specimens (20%) fell outside the 95% range found in the histologically normal specimens.

Telomere DNA content in a test cohort of breast tumors

TC next was determined in a cohort of 27 DCIS, 104 Stage I and 32 Stage IIA breast tumors**.** In contrast to the histologically normal group, there was a wide range of TC distribution in the tumor specimens within the test cohort (Fig. 1). Of the 27 DCIS cases, 10 (37%) fell outside the normal range. Similarly, 44 of the 104 Stage I tumors (42%) and 14 of the 32 Stage IIA tumors (44%) fell outside the normal range. However, the DCIS specimens as a group had longer telomeres than the Stage I ($P = 0.0152$) and Stage IIA ($P = 0.0338$) tumors.

Telomere DNA content in a validation cohort of breast tumors

The results were validated in an independent population-based breast tumor cohort comprised of 494 specimens. TC was determined in 95 DCIS, 244 Stage I, 112 Stage IIA, 39 Stage IIB and 4 Stage IIIA breast tumors**.** Fifty-five of the 95 DCIS cases (58%), 127 of the 244 Stage I (52%), 65 of the 112 Stage IIA (58%), 20 of the 39 Stage IIB (51%) and 3 of the 4 Stage IIIA (75%) tumors fell outside of the

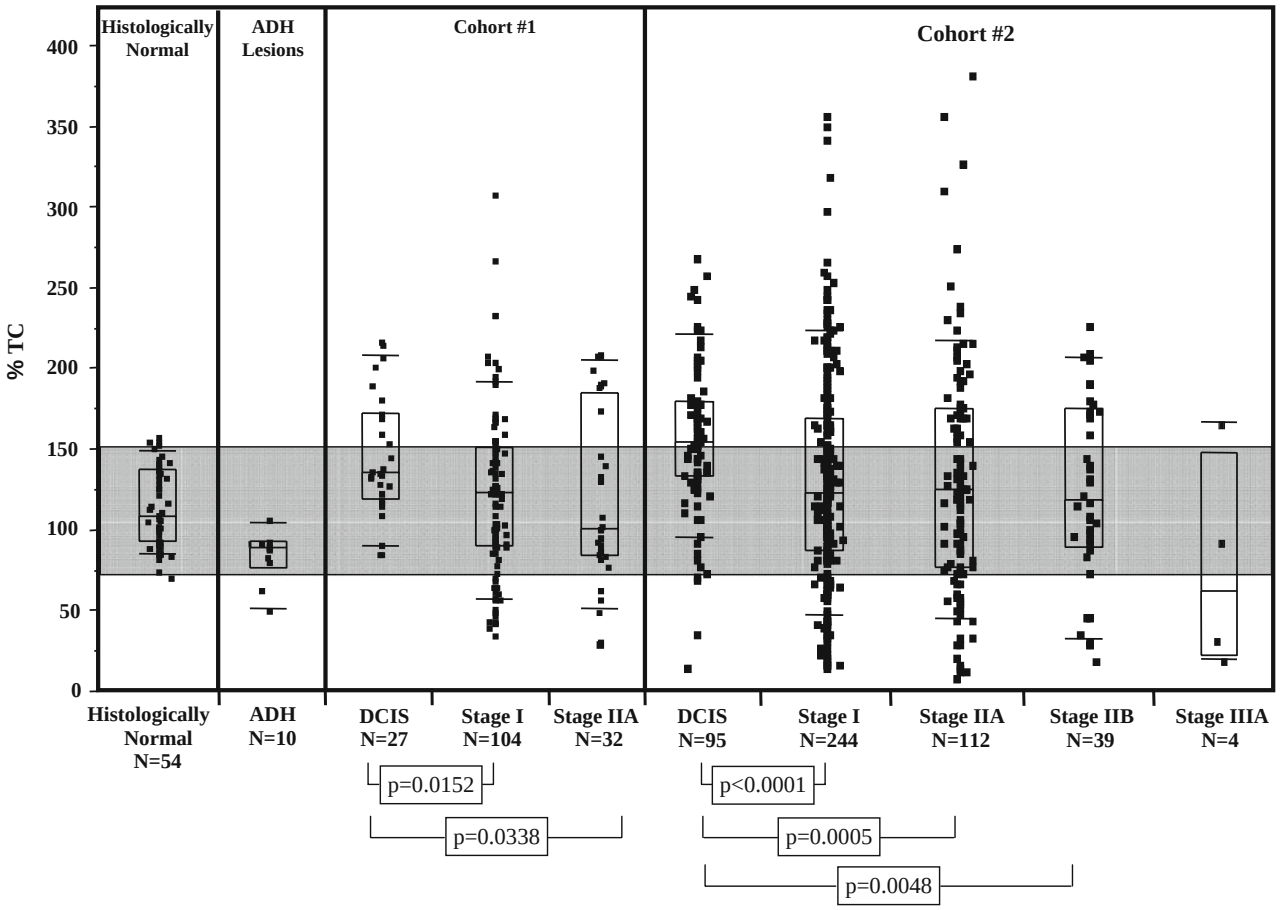

Fig. 1 TC distributions in histologically normal breast tissues derived from reduction mammoplasties, ADH lesions, and two independent cohorts of DCIS lesions and invasive breast carcinomas (Stage I–III). The numbers of tissues analyzed are indicated (*N*). TC is expressed as a ratio of TC in a placental DNA control. The boxes represent group medians (line across middle) and quartiles (25th and 75th percentiles) at its ends. Lines above and below boxes indicate 10th and 90th percentiles, respectively. The gray shaded area indicates 95% of TC measurements in the histologically normal group (75–154%). *Note*: Although the individual data points are horizontally shifted, some are still overlapping and therefore may not be visible

normal range defined by the histologically normal breast tissues. Again, the DCIS group had longer telomeres than the Stage I ($P < 0.0001$), Stage IIA ($P = 0.0005$) and Stage IIB ($P = 0.0048$) tumors (Fig. 1). In both the test and validation cohorts, TC did not correlate with ethnicity, nodal status, or ER and PR status.

Extent of AI in histologically normal tissue and ADH lesions

To extend and confirm these findings, AI, another independent marker of genomic instability, was measured and compared in the same tissue cohorts. The mean number of sites of AI was 0.26 in the histologically normal and 1.00 in the ADH groups (Fig. 2). As compared to the histologically normal group, the ADH group showed a significant increase in the extent of AI ($P = 0.0002$), although the small number of ADH specimens must be noted.

Extent of AI in a test cohort of breast tumors

Next, the extent of AI was analyzed in the test cohort. The mean number of sites of AI was 2.63 in DCIS, 3.24 in Stage I tumors and 2.84 in Stage IIA tumors (Fig. 2). All groups were statistically different when compared to the histologically normal group ($P < 0.0001$ for each). As observed for TC, there was no difference in the extent of AI in the DCIS group compared to any of the invasive groups. Additionally, there was no difference between Stage I and Stage IIA tumors.

Extent of AI in a validation cohort of breast tumors

These findings were replicated in the validation cohort. The mean number of sites of AI was 3.03 in DCIS, 3.08 in Stage I, 2.98 in Stage IIA, 2.92 in Stage IIB and 3.50 in Stage IIIA (Fig. 2). All categories were statistically different from the histologically normal group ($P < 0.001$ for each). There was no statistically significant difference between the DCIS group and the groups of invasive carcinoma or between any of the invasive groups. Additionally, there was no statistical difference in the mean number of sites of AI between paired groups by stage between the test and validation cohorts of breast tumors. Next, we tested our previously operationally-defined threshold for AI (i.e. $\geq$2 sites of AI) in these tissue cohorts [39]. Using this threshold, 0 of the 54 (0%) histologically normal breast specimens contained $\geq$2 sites of AI (Table 2). In contrast, 131 of the 163 tumors in the test cohort (80.4%) and 402 of the 494 tumors in validation cohort (81.4%) contained $\geq$2 sites of AI (Table 2). AI did not correlate with ethnicity, nodal status, or ER and PR status in both the test and validation cohorts.

Allelic frequency in DCIS and invasive tumors

Since the mean number of sites of AI in specimens of DCIS was nearly identical to the invasive tumors in both study cohorts, we next determined whether there was a difference in the allelic frequencies at each locus as a function of stage of progression. Since the individual loci have no known involvement in the development of breast cancer, there should be no selection pressure and the frequency of AI at a particular locus should not differ as a function of

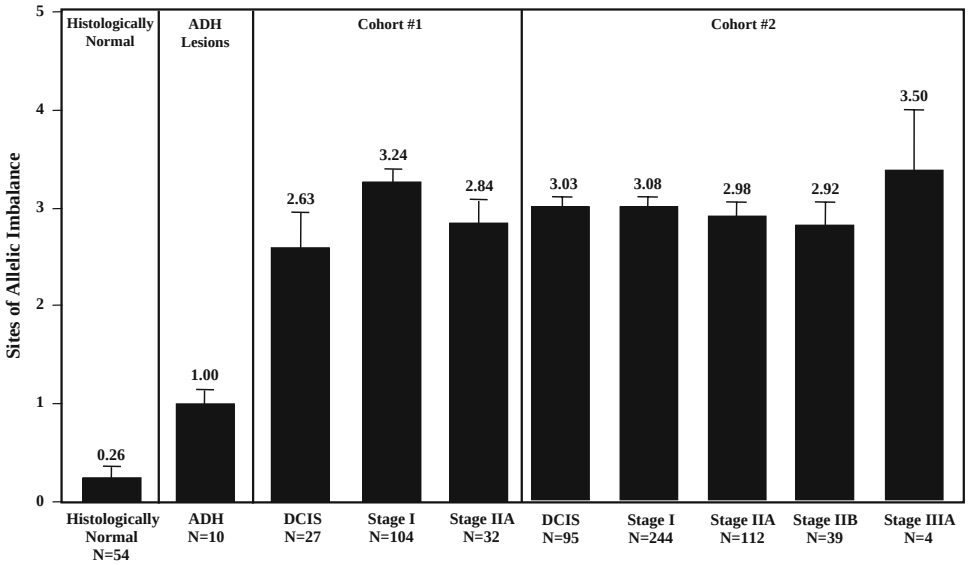

Fig. 2 Extent of allelic imbalance in histologically normal breast tissues derived from reduction mammoplasties, ADH lesions, and two independent cohorts of DCIS lesions and invasive breast carcinomas (Stage I–III). The numbers of tissues analyzed are indicated (*N*). The bars indicate the mean number of unbalanced loci (shown for each group) ± standard errors. Abbreviations: ADH, atypical ductal hyperplasia; DCIS, ductal carcinoma in situ

Table 2 Extent of AI in cohorts of breast tissue

Group	*N*	# Samples ≥2 AI	%
Histologically normal	54	0	0.0
ADH	10	1	10.0
Cohort #1			
DCIS	27	20	74.1
Stage I	104	92	88.5
Stage IIA	32	19	59.4
Combined	163	131	80.4
Cohort #2			
DCIS	95	81	85.3
Stage I	244	196	80.3
Stage IIA	112	89	79.5
Stage IIB	39	32	82.1
Stage IIIA	4	4	100.0
Combined	494	402	81.4

Table 3 Frequency of AI at distinct loci in DCIS and invasive tumors

Loci designation	DCIS ($N = 122$)	Invasive ($N = 535$)	*P* value
D8S1179	0.19	0.26	0.108
D21S11	0.25	0.31	0.207
D7S820	0.03	0.08	0.066
CSF1PO	0.04	0.06	0.377
D3S1358	0.30	0.21	0.052
TH01	0.39	0.28	0.014
D13S317	0.18	0.24	0.175
D16S539	0.17	0.24	0.086
D2S1338	0.17	0.13	0.284
D19S433	0.14	0.18	0.310
vWA	0.35	0.32	0.538
TPOX	0.25	0.23	0.572
D18S51	0.10	0.10	1.000
D5S818	0.22	0.27	0.277
FGA	0.12	0.15	0.512

progression. For this analysis, the DCIS and invasive tumors were combined from the two tumor cohorts. As shown in Table 3, there were no statistically significant differences in the allelic frequencies of 14 of the 15 markers between the DCIS and invasive groups, except at the TH01 locus which showed an increase in AI in the DCIS samples compared to the invasive tumors ($P = 0.014$).

Discussion

The accumulation of genomic instability is characteristic of all carcinomas, including breast [1]. It has been proposed that breast cancer progression can be modeled as a sequence of events progressing from normal epithelium to ADH, to DCIS, to finally the development of an invasive breast carcinoma [2, 3]. However, the genetic changes that underpin these histological changes still remain to be fully understood.

In this investigation we used TC and AI, two independent quantitative markers of genomic instability, to demonstrate that genomic instability increases as a function of the extent of breast disease (i.e. histologically normal tissue to ADH to DCIS). Alterations in TC and the extent of AI plateau in DCIS and do not increase further with increasing stage in invasive carcinomas. However, TC measurements show further telomere shortening between DCIS lesions and invasive carcinomas. The later finding is consistent with our previous studies demonstrating low TC compared to high TC confers an adjusted relative hazard of 4.43 (95% CI 1.4–13.6, $P = 0.009$) [22] in a cohort of 77 women. Additionally, in a population-based study of 530 women, low TC conferred an adjusted relative hazard of 2.88 (95% CI = 1.16–7.15; $P = 0.022$) [23].

Our TC findings are consistent with our previous reports that TC correlates with Stage in invasive carcinomas [22]. Here, we show that 95% of the histologically normal breast tissues analyzed in this study fall within a range of 75–154% of the placental DNA control, nearly identical to the range previously reported [21], demonstrating that TC is tightly regulated regardless of inherent tissue properties that may affect TC, such as organ site or patients' age. However, evidence of telomere dysregulation (i.e. attrition or elongation) was present in all the tumor cohorts. Speculatively, the finding of telomere elongation in tumors reflects the reactivation of telomerase, which is reactivated in 85–90% of tumors [40]. However, the extent of reactivation varies amongst tumors as demonstrated by Hines and colleagues who showed an approximate 800-fold difference in telomerase expression among a panel of 36 breast tumors [41]. Additionally, it has been postulated that early telomerase activation results in longer telomeres as compared to late activation, thus providing an opportunity for continued telomere shortening and accumulation of genomic instability.

Our observations confirm and extend the results of Ellsworth et al. [35] which demonstrated that levels of genomic instability are equivalent in DCIS lesions and advanced invasive tumors. However, that particular study utilized a panel of markers that were previously identified as important genes in the development of breast cancer. This confounds the ability to clearly interpret AI across these markers as genomic instability since these markers may be linked to oncogenes or tumor suppressor genes involved in the development of breast cancer. In contrast, the assay used in this study is based on AI at 16 random

microsatellite regions that have no known involvement in the development of breast cancer, and thus reflect genomic instability independent of their linkage to genes involved with breast tumorigenesis. The differences in the extent of imbalance among the particular loci may reflect the proximity of the microsatellite region to the telomere ends. Chromosomal differences in telomere length may also contribute to the individual heterogeneity.

In conclusion, the level of genomic instability assessed by (i) dysregulation in TC (i.e. outside the 95% range found in normal breast tissue) and (ii) extent of AI assessed at 16 microsatellite loci located throughout the genome, increases along the continuum of breast disease from histologically normal, to ADH lesions to DCIS and the level of genomic instability did not differ between DCIS and invasive carcinomas. In all, these findings suggest that DCIS lesions have the same extent of genomic instability (i.e. TC alterations and increased AI) as invasive carcinomas; thus supporting the notion that invasive carcinomas evolve from or in parallel with DCIS.

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